Identification and characterization of molecular genetic bases responsible for the predisposition to chronic mucocutaneous candidiasis in humans

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Thèse

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Identification et caractérisation des bases génétiques moléculaires responsables de la prédisposition à la candidose cutanéo-muqueuse chronique chez l'homme

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Résumé

Mon projet de thèse a consisté en l'identification et la caractérisation moléculaire et immunologique de patients présentant une susceptibilité accrue aux infections fongiques par *Candida* sp. dans le syndrome Mendélien de candidose cutanéo-muqueuse chronique (CCMC).

La CCMC est caractérisée par des infections persistantes ou récurrentes de la peau, des ongles et des muqueuses par les champignons *Candida*, principalement *C. albicans.* Elle est fréquemment associée à d'autres infections opportunistes dans certaines immunodéficiences primaires ou acquises, ou bien elle peut être associée à un tableau auto-immun. La CCMC peut finalement être isolée (CCMCi) sans autre tableau clinique sévère: la plupart des cas rapportés sont sporadiques, mais il existe également des cas familiaux avec une hérédité mendélienne autosomique principalement dominante (AD) ou plus rarement récessive (AR).

Basés sur les données de la littérature, qui démontrent un rôle majeur de l'immunité dépendante des IL-17s dans la résistance aux infections mucocutanées vis-à-vis de *C. albicans* et nos résultats récents, qui démontrent un défaut de cette immunité dans certaines immunodéficiences primaires associées à une CCMC [les syndromes AD-HIES et AR APS-1, ainsi que chez les patients déficients en CARD9, nous avons émis l'hypothèse que parmi les patients atteints de CCMCi, certains pourraient présenter un défaut génétique affectant spécifiquement l'immunité IL-17-dépendante.

Au début de ma thèse, j'ai participé à l'identification des deux premières étiologies génétiques de la CCMCi : le défaut autosomique récessif (AR) complet en IL-17RA et autosomique dominant (AD) en IL-17F. Plus récemment, j'ai identifié la troisième et la plus fréquente étiologie génétique de la CCMC par l'identification de mutations gain de fonction dans le gène STAT1 suite à une approche explorant l'ensemble du génome (séquençage de l'ensemble des exons). Ces mutations engendrent une « hyper-réponse » aux interférons de type I et II et à l'IL-27 qui inhibent la différentiation des lymphocytes T sécréteurs d'IL-17, impliqués dans l'immunité mucocutanée vis-à-vis de *C. albicans* chez l'homme.

En conclusion, nous avons identifié, en 2011, des trois premières étiologies génétiques de la CCMCi, avec les défauts AR en IL-17RA, AD en IL-17F et des mutations gain-de-fonction de *STAT1,* toutes associées à un défaut de l'immunité dépendante de l'IL-17. Des mutations gain-de-fonction de *STAT1* représentent à ce jour la cause génétique la plus fréquente de la CCMCi avec au total 94 patients rapportés dans la littérature depuis 2011. Nous avons ainsi démontré que la CCMCi est une immunodéficience primaire, associée à un défaut de l'immunité réalisée par les IL-17s. Ces travaux ont des implications majeures dans le domaine immunologique avec la description et la caractérisation des mécanismes biologiques impliqués dans l'immunité protectrice spécifique de *C. albicans* et une meilleure compréhension des mécanismes physiopathologiques associés à une susceptibilité accrue aux infections fongiques, dans des conditions naturelles d'infection ; et dans le domaine médical, avec la possibilité de diagnostics moléculaires, un conseil génétique en cas de diagnostic positif, une meilleure prise en charge des patients.

Summary

My project consists in the molecular and immunological identification and characterization of patients with increased susceptibility to fungal infections with *Candida* sp. suffering from the Mendelian syndrome of chronic mucocutaneous candidiasis (CMC).

CMC is characterized by persistent or recurrent infections of the skin, nails and mucosae by *Candida* fungi, especially *C. albicans*. CMC is frequently associated with other opportunistic infections in some acquired or primary immunodeficiencies, or can be associated with autoimmune disorders. Finally, CMC may be present as an isolated form (chronic mucocutaneous candidiasis disease or CMCD) without any other severe infectious or autoimmune clinical manifestation: most reported cases are sporadic, but there are also familial cases with autosomal dominant (AD) or recessive (AR) Mendelian inheritance. Based the literature, which demonstrated a major role of IL-17 cytokines in mucocutaneous immunity with *C. albicans*, and our recent results, which show an impairment of IL-17 immunity in some primary immunodeficiencies associated with CMC (AD-HIES syndrome, AR APS-1, and CARD9-deficient patients), we hypothesized that among CMCD patients, some might have a genetic defect affecting specifically the IL-17-dependent immunity.

At the beginning of my PhD, I participated in the identification of the first two genetic etiologies of CMCD: complete AR IL-17RA and partial AD IL-17F deficiencies. More recently, I identified the third and most common genetic etiology of CMCD by identifying gain of function mutations in the *STAT1* gene following an approach exploring the whole genome (sequencing of all exons). These mutations are responsible for a "hyper-response" to type I and II interferons and IL-27, which inhibit the differentiation of IL-17-producing T cells. Impaired IL-17 immunity results in reduced mucocutaneous defenses against *C. albicans* in humans.

In conclusion, we have identified in 2011, the first three genetic etiologies of CMCD with AR IL-17RA and AD IL-17F deficiencies and gain-of-function *STAT1* mutations, all associated with an impaired IL-17-dependent immunity. Gain-of-function *STAT1* mutations represent the most frequent genetic cause of CMCD with a total of 94 patients reported in the literature since 2011. We have shown that CMCD is a primary immunodeficiency associated with inborn errors of IL-17 immunity. This work has important implications in the field of immunology with the description and characterization of the biological mechanisms involved in protective immunity specific to *C. albicans* and a better understanding of the pathophysiological mechanisms associated with increased susceptibility to fungal infections in natural conditions of infection, and in the medical field, with the possibility of molecular diagnostics, genetic counseling for a positive diagnosis, and a better follow-up of the patients.

Contents

List of publications

1. Puel A*, Cypowyj S*, Bustamante J, Wright JF, **Liu L**, Lim HK, Migaud M, Israel L, Chrabieh M, Audry M, Gumbleton M, Toulon A, Bodemer C, El-Baghdadi J, Whitters M, Paradis T, Brooks J, Collins M, Wolfman NM, Al-Muhsen S, Galicchio M, Abel L, Picard C, Casanova JL. Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. **Science**. 2011 Fev 24 332:65–68.

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3. Okada S*,Cypowyj S*,**Liu L***, Kong XF*, Kreins AY, Mekki N,Toubiana J,Hiller J,Okada C, Boisson B, Morel JD, Soltész B, Tóth B, Bensifi M, Toulon A, Gulácsy V, Schnopp C, Schaller M, Wijaya-Kusuma T, Jansson A, Sawalle J, Marie-Cardine A, Bue M, Drexel B, Hoernes M, Bustamante J, Firinu D, Meuwissen H, Grimprel E, Karmochkine M, Weiss L, Becerra J, Lagos M, Fouyssac F, Wallace MR, Lortholary O, Tucker M, Willis M, Leonard S, Ferdman R, Church J, Fieschi C, Suarez F, Hermine O, Kerns L, Yuenyongviwat A, Polak M, Bodemer C, Reich K, Debre M, Belohradsky B, Dupont B, Roesler J, Bousfiha A, Sanal O, Fischer A, Blanche S, Muhsen S, Kobayashi M, Reichenbach J, Seger R, Klein C, Renner E, Abel L, Traidl-Hoffmann C, Picard C, Maródi L, Boisson-Dupuis S, Puel A and Casanova JL. Gain-of-function STAT1 mutations underlying CMC: enhanced responses to IFN-α/β, IFN-γ, and IL-27 impair IL-17 T-cell immunity. **S**ubmitted.

4. Lanternier F*, Pathan S*, Vincent Q, **Liu L**, Cypowij S, Prando C, Migaud M, Taibi L, Ammar-Khodja A, Stambouli OB, Guellil B, Merad-Boudia A, Jacobs F, Goffard JC, Shepers K, Marmol V, Bachelez H, Michel L, Lefranc G, Fraitag S, Bougnoux ME, Lortholary O, Abel L, Jean-Laurent Casanova JL*, Picard C*, Grimbacher B* and Pue1 A*. Homozygous CARD9 mutations in patients with invasive dermatophytic disease. **N Engl J Med**, in press.

*** equal contribution**

Abbreviation

AD	Autosomal dominant
AD-HIES	Autosomal dominant hyper-immunoglobulin E syndrome
AMP	Antimicrobial peptide
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APS-1	Autoimmune polyendocrine syndrome type 1
AR	Autosomal recessive
CCD	Coiled-coil domain
CEPH	Centre d'étude du polymorphisme humain (Center for the Study of Human Polymorphisms)
CLR	C-type lectin receptor
CMC	Chronic mucocutaneous candidiasis
CMCD	Chronic mucocutaneous candidiasis disease
DC-SIGN	Dendritic cell specific ICAM-3-grabbing non-integrin
DBD	DNA-binding domain
GAS	Gamma interferon activated Sequence
GOF	Gain-of-function
ISRE	Interferon Stimuated Responses Element
LOF	Lost-of-function
MR	Mannose receptor
PBMC	Peripheral blood mononuclear cell
PID	Primary immunodeficiency
PRR	Pattern recognition receptors
SCID	Severe combined immunodeficiency
STAT	Signal transducer and activator of transcription
TAD	Transactivation domain
TLR	Toll-like receptor

1. Introduction

The presence of a pathogen in the environment is an indispensable but not always a sufficient factor for the development of an infectious disease. A complex interplay between environmental (microbial and non-microbial) and human (non-genetic and genetic) factors determines immunity to infection and the resulting clinical outcome of infection. Accumulating evidences suggest that human genetic factors play a particularly important role in immunodeficiencies and susceptibility to infectious diseases [1-3]. According to the dominant paradigm, genetic predisposition to infectious diseases segregates in either a Mendelian or polygenic pattern of inheritance [2]. Following a Mendelian predisposition model, as of November 2011, more than 180 molecularly defined syndromes associated with abnormal immune function have been identified in more than 200 primary immunodeficiency diseases (PIDs) and new entities are reported almost every month [4]. Typically, these disorders are individually rare and confer predisposition to multiple infectious diseases (one gene, multiple infections). They affect immune responses in various ways, typically, but not exclusively, involving hematopoietic cells such as neutropenia, a lack of B or T cells. It gradually became clear that not all PIDs confer predisposition to multiple infections, or even recurrent infections. An increasing number of disorders are known to confer Mendelian predisposition to a single type of infection (one gene, single infection) [5]. We focus on the genetic determinism of such infectious diseases, and test the hypothesis that severe infections arising in otherwise healthy children may result from Mendelian (monogenic) genetic errors. We adopted candidate gene and hypothesis generating approaches to reveal Mendelian genetic etiologies for a fungal infectious disease: chronic mucocutenous candidiasis (CMC).

2. Chronic mucocutaneous candidiasis (CMC)

2.1 Epidemiological and clinical data

Candidiasis is a fungal infection caused by *Candida* species and more commonly by *Candida albicans* [6]. *C. albicans* is a commensal organism of the mucous membranes of the oral cavity, gastrointestinal tract and vulvovaginal walls, and the prevalence of asymptomatic is approximately 50% in the general population. However, in some individuals (immunocompromised individuals, newborns, the elderly), *C. albicans* becomes a major opportunistic pathogen causing two diseases generally exclusive: invasive candidiasis (systemic candidiasis, which is often acute) or superficial candidiasis of the skin, nails and mucous membranes (mucocutaneous candidiasis, which is usually chronic, therefore it is called chronic mucocutaneous candidiasis or CMC) [7, 8]. Innate or acquired granulocyte defects are often responsible for systemic candidiasis, while innate or acquired T cell deficiencies are often responsible for mucocutaneous candidiasis [7-9]. My thesis focused on CMC.

CMC is a clinically highly heterogeneous disease, mainly characterized by severe, chronic or recurrent infections of the skin, nails and mucosae by the yeast *Candida*, mainly *C. albicans*. [8, 10, 11]. This disease might be severely disabling in particular due to debilitating esophageal stricture with potential mal-digestion/-absorption. In addition, it can be life threatening as the chronic infections are associated with the development of squamous cell carcinomas of the oral cavity or of the esophagus or with intracranial aneurysms [12-17]. A number of CMC patients also develop accompanying autoimmune disorders, mainly of the thyroid, suggestive of an underlying deregulation of the immune system.

It has long been recognized that protection from mucocutaneous candidiasis relies on cell-mediated immunity [18, 19]. Indeed, severe oro-pharyngeal candidiasis was among the most common opportunistic infections in HIV-infected patients, and represented a marker of disease progression before the introduction of effective antiviral treatments [18, 20]. Patients with severe combined immunodeficiency (SCID) or combined immunodeficiency (CID) and impaired numbers and/or function of T lymphocytes,

often suffer from persistent mucocutaneous infections with *C. albicans* [4, 5, 21]. CMC is also common in patients receiving immunosuppressors, steroids or antibiotics [8]. However, CMC is one among many other opportunistic infections occurring in these individuals who are vulnerable to a large spectrum of pathogens.

In contrast, CMC is one of the major infections found in autosomal dominant hyper-immunoglobulin E syndrome (AD-HIES). It is a complex PID characterized by high serum IgE levels, severe *Staphyloccocus aureus* pulmonary infections, recurrent staphylococcal skin abscesses and impaired inflammatory responses, referred to as cold abscesses, and skeletal abnormalities [22-24]. Classic AD-HIES is caused by dominant-negative mutations in *STAT3* [25]. The transcription factor STAT3 (Signal Transducer and Activator of Transcription 3) is implicated in signaling pathway of a large number of cytokines, mainly those belonging to the gp130 family (IL-6, IL-11, OSM, LIF, CNTF, NNT-1, G-CSF, CT-1) and the IFN family (IL-10, IL-20, IL-22). It can also be activated by IFN-α/β, common gamma-chain family cytokines (IL-2, IL-7, IL-9, IL-15, IL-21), single chain family cytokine (GH) and receptor tyrosine kinases (EGF, PDGF, CSF-1, HGF) [25, 26]. An autosomal recessive (AR) form of susceptibility to CMC has been associated with a homozygous premature stop codon mutation in CARD9 found in a large consanguineous Iranian family. Patients of this family had recurrent superficial fungal infections with *Candida* sp. and dermatophytes, and/or invasive infection of the brain with *Candida* sp. [27]. The protein CARD9 is a key adapter in the signaling pathway of C-type lectin receptors (CLRs) such as Dectin-1, Dectin-2 and MINCLE [28-30], which are pattern recognition receptors (PRRs) critical for immune responses to fungi, including *C. albicans* [31-35].

CMC is one of the hallmarks of the autoimmune polyendocrine syndrome type 1 (APS-1), also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) syndrome [36]. Autosomal recessive APS-1 (AR APS-1) is due to biallelic mutations in the autoimmune regulator (*AIRE*) gene which result in T cell self-tolerance impairment [37, 38]. Present in over 90% of APS-1 patients, CMC is usually the earliest and only infectious clinical manifestation, but the underlying

immunodeficiency remained puzzling for a long time [21, 24, 39-43]. CMC was also described in combination with thyroid disease, segregating as an AD trait [21, 42-46]. From now on, throughout the text, I will reference the CMC associated with these conditions (AD-HIES, AR APS-1, AD CMC with thyroiditis, or AR CARD9 deficiency) as syndromic CMC.

Finally, CMC can exist as an isolated form (CMC disease or CMCD), arising in otherwise healthy individuals without any other overt infectious or autoimmune clinical manifestation [8, 10, 11, 21]. However, invasive candidiasis, dermatophytosis, bacterial infections of the lungs and skin (mainly by *S. aureus*) [47-49], or severe viral disease [50] have been reported in some patients [21]. In addition, an increasing number of CMCD patients with autoimmune, especially thyroid, diseases are reported [21]. Finally, CMCD is associated with an increased risk of developing oral or esophageal squamous cell carcinoma and cerebral aneurysms, which origin remains unknown [21]. It is a rare disease, which most often appears in early childhood, with a prevalence estimated at 1 out of 100,000 individuals.

The first CMCD cases were described during the 1960s, familial forms with mainly AD segregation, or more rarely AR inheritance in some consanguineous families, have been reported [8], suggesting a genetic predisposition to CMCD [21]. As many sporadic and familial cases have been described [21], it was suggested that CMCD could result from Mendelian genetic defects, at least for some patients [21]. Despite a large number of CMCD patients studied during the 1980s, no genetic etiology or robust immunological phenotype could be identified. Then, with the development of antifungal therapies, research on CMCD gradually declined. Recently, studies have strongly progressed in the identification and the characterization of genetic and physio-pathological mechanisms of CMC either syndromic or isolated.

2.2 Syndromic CMC: a role of IL-17 immunity?

In recent years, the search for the genetic predisposition to CMC was boosted by the development of oropharyngeal candidiasis in mouse models [51], the characterization of "Th17 cytokines" (IL-17A, IL-17F and IL-22) [52, 53] and finally the characterization of the pathogenesis of primary immunodeficiencies associated with CMC [21, 23, 24, 41-43, 46, 54, 55].

2.2.1 IL-17 immunity

IL-17 and Th17 CD4 T cells are believed to confer protection against fungal pathogens including *C. albicans* in mice [9, 42, 51] and humans [52, 56]. In addition, some studies in mice and humans have demonstrated that IL-17 and Th17 also play a protective role against intracellular bacteria like *Listeria monocytogenes, Salmonella enterica*, or *Mycobacterium tuberculosis* [57](Table 1).

Table 1: IL-17 immunity in immunity against bacterial, mycobacterial or fungal pathogens [50]

Class	Pathogen	Protection	Effects of IL-17	Host studied
Bacteria	*Bacteroides fragilis*	No	Contributes to intra-abdominal abscess formation	Mice [58]
	Bordetella pertussis	Yes	Required for vaccine-primed protection	Mice [59]
	Borrelia sp.	No	Contributes to the development of arthritis	Mice [60]
	Citrobacter rodentium	Yes	Increases survival	Mice [61]
	Escherichia coli	Yes	Reduces bacterial burden	Mice [62]
	Helicobacter pylori	No	Associated with chronic gastric inflammation	Human [63, 64]
	Klebsiella pneumoniae	Yes	Reduces bacterial burden, increases survival	Human [65], mice [66]
	Listeria monocytogenes	Yes	Reduces bacterial burden in liver, contributes to granuloma formation	Mice [67, 68]
	Mycoplasma pneumoniae	Yes	Enhances the kinetics of bacterial clearance	Mice [69]
	Porphyromonas gingivalis	Yes	Prevents periodontal bone destruction	Mice [70]
	Pseudomonas aeruginosa	No	Associated with pulmonary exacerbations in patients with cystic fibrosis	Human [71], mice [72]
	Streptococcus pneumoniae	Yes	Prevents colonization	Mice [73]
	Salmonella enterica	Yes	Reduces bacterial burden	Mice [74]
Mycobacteria	*Mycobacterium tuberculosis*	Yes	Enhances T helper type 1 memory response, reduces mycobacterial burden after vaccination	Mice [75]
	M. bovis, bacille de Calmette–Guerin (BCG)	Yes	Contributes to acute neutrophil-mediated inflammation and granuloma formation	Mice [75]
Fungi	*Aspergillus fumigatus*	No	Increases fungal burden (intranasal inoculation)	Mice [76]
	C. albicans	Yes	Reduces fungal burden, increases survival (intravenous inoculation or natural cutaneous infection)	Mice [77], human [78]
	C. albicans	No	Increases fungal burden (intragastric inoculation)	Mice [76]
	Cryptococcus neoformans	Yes	Reduces fungal burden, increases survival	Mice [79]
	Pneumocystis carinii	Yes	Reduces fungal burden	Mice [80]

2.2.1.1 Pattern recognition receptor (PRR)-mediated fungal recognition

C. albicans cell wall is a complex array of layered proteins and carbohydrates. Mannan and manoproteins compose the outer portion, and β-(1,3)-glucan and chitin moieties compose the inner layer. *C. albicans* is a dimorphic fungus switching between yeast and hyphal forms. Expression of cell wall proteins and carbohydrates is significantly altered during transition of these two forms. The innate immune system recognizes components of the *C. albicans* cell wall, distinguishes fungal forms and directs skewing of Th cell responses by pattern recognition receptors (PRRs). PRRs capable of recognizing fungal components are identified in several PRR families: Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and Complement receptor 3 (CR3) [81]. Of the TLRs, TLR2 and TLR4 are the major participants in *C. albicans* recognition. TLR2 binds to phospholipomannans and β-glucan (the major component of yeast zymosan) and acts in combination with Dectin-1 (a CLR receptor) to induce proinflammatory responses in a variety of *Candida* infection settings [82]. TLR4 recognizes *C. albicans* O-linked mannan and stimulates production of the inflammatory cytokine TNF-α in human mononuclear cells and murine macrophages [83]. CLRs appear to be more critical than TLRs in *C. albicans* recognition. Several extracellular and transmembrane CLRs, including the mannose receptor (MR), Dectin-1, Dectin-2, dendritic cell specific ICAM-3-grabbing non-integrin (DC-SIGN) and the collectins, are involved in antifungal immunity [84]. Although their roles need to be further elucidated, Dectin-1, dectin-2 and MINCLE are the best-characterized CLRs with respect to *Candida*. Those receptors recognize different antigens presented dynamically by either the yeast or hyphal form. Dectin-1 recognizes the β-(1,3)-glucan, which is usually buried underneath a layer of cell wall proteins and mannan moieties, posing an issue of accessibility for innate immune cells. Nevertheless, β-glucan is exposed in bud scars that are revealed during the process of hyphal transition, which facilitates its recognition and may be the essential signal that alerts the host of a transition from fungal colonization to infection [28]. Dectin-2 recognizes N-linked mannan sugars, which are localized in the exterior layer of the yeast cell wall [29], and appears to be especially important in recognition of hyphae [30]. In addition to Dectins, MINCLE and the mannose receptor (MR) recognize

mannan carbohydrates from *Candida*. In human peripheral blood mononuclear cells (PBMCs), mannans were found to induce more IL-17 than other fungal components such as β-glucan and chitin [85]. Although *in vivo* studies have not yet fully clarified which CLR is more important, details of their respective signaling pathways are progressively elucidated. They appear to mediate signaling through the Syk kinase, the adaptors CARD9/Bcl-10/MALT1, and the NF-κB and Ras/Raf-1 pathways [86, 87]. Ultimately, it is likely that signaling through a multiplicity of PRRs that recognize different components of *C. albicans* is needed to develop an optimal immune response. This combined pathways trigger expression and secretion of IL-6, IL-23, and IL-1β, which together with TGF-β induce naïve T cell differentiation towards Th17 cells via the activation of the transcription factors STAT3 downstream of IL-6 and IL-23 and ROR-γt [88].

2.2.1.2 Th17 differentiation

The T helper cell (Th) paradigm, introduced by Mosmann and Coffman more than two decades ago, has been used to explain how different adaptive immune responses are elicited in the host organism for the purpose of eradicating infections with diverse microbial pathogens [89]. Th1 and Th2 cells described in the original studies, have now been joined by Th17 cells who produce high levels of IL-17A, IL-17F, and IL-22 [90-92]. Later on, some studies distinguished high IL-22-producing T cells from Th17 and named them Th22 [93, 94]. These cells are involved in clearance of extracellular bacteria and fungi, particularly at mucosal surfaces [61, 95, 96]. Another subset of CD4+ T cells, the regulatory T cells (Tregs), has also emerged suppressing effector T cell responses thereby preventing their potentially pathogenic effects [97]. The T helper cell differentiation program is largely controlled by cytokines produced in response to microbial products by innate immune cells. These include IL-12/IFN-γ for Th1 differentiation, IL-4 for Th2 differentiation and TGF-β to induce Tregs (iTregs). Th17 differentiation program is more complex, and there are some discrepancies between human and mouse Th17 cell differentiation. The first one is the role of TGF-β. Indeed, TGF-β has been shown to be essential for Th17 cell differentiation in mice. In human, it shows a dose-dependent effect: an inhibitory effect at high doses and an inducing effect at low

doses. However, the presence of TGF-β is necessary but not sufficient for Th17 cell differentiation initiation. This process requires at the same time the presence of IL-6 in mice and pro-inflammatory cytokines such as IL-1, and IL-21 in human. Then, IL-23 functions both in mice and human, at a late stage of Th17 cell differentiation, for their expansion and maintenance [98-100].

The differentiation of each effector T cell subset requires the induction and/or function of a series of transcriptional regulators that interact with each other in complex networks and thus orchestrate the functional program of the cells [101]. Each T helper cell differentiation program is pivoted by their "master regulators": T-bet for Th1 cells, GATA3 for Th2 cells [102], Foxp3 for Tregs, and Retinoid-related orphan receptor (ROR)-γt (ROR-γt for mice and its homologue RORC for human) for Th17 [103]. Th17 shares with iTreg cells an early common transcriptional programming which requires TGF-β. TGF-β signaling leads to an early transient co-expression of the lineage-defining transcription factors Foxp3 and ROR-γt. Foxp3 represses ROR-γt in a TGFβ dose dependent manner: high doses of TGF-β repressed ROR-γt via increased Foxp3 and drive iTreg differentiation, whereas low doses of TGF-β, in cooperation with Th17-promoting cytokines including IL-6, IL-21 and IL-23 that activate STAT3, override the Foxp3-mediated repression of ROR-γt, and favor Th17 differentiation [100]. In contrast to Th1 and Th2 differentiation, during late lineage specification, Th17 present an alternative developmental program than the one used during early differentiation. Functional maturation and maintenance of Th17 require exposure to IL-23, which receptor IL23R is expressed only in activated ROR-γt+ Th17 cells but not in naïve CD4 T cells. IL-23 is indispensable for maintaining and stabilizing the expression of Th17 cell signature genes including *Ror-γt* (mouse) or *RORC* (human), *IL17*, and *IL23R*, while repressing genes that destabilize Th17 cells such as *IL2* and *IL27R* [99].

Since tissue damage can be impacted due to over-induction of inflammatory pathways by Th17 [92], their generation is strictly regulated. The earliest documented biological activity of IL-17 was its effects on synoviocytes from patients with rheumatoid arthritis [104]. IL-17 activity was immediately linked to inflammation by inducing the production of IL-6 and IL-8, as they lead to fever, acute phase responses

(caused by IL-6) and the accumulation of neutrophils in blood and tissue (caused by IL-8) [104]. IL-17 activity also contributes to chronic inflammation [105]. For example, it causes matrix destruction by inhibiting matrix production of chondrocytes and osteoblasts. IL-17 and Th17 have been associated with an increasing number of chronic inflammatory diseases including rheumatoid arthritis, psoriasis and psoriatic arthritis, ankylosing spondylitis, Crohn's disease, multiple sclerosis, vasculitis and atherosclerosis, lung disorders, asthma and chronic obstructive pulmonary disease and chronic obstructive pulmonary disease [106].

As Th17 perform both protection and inflammatory effect, their generation is strictly regulated [90]. The negative regulatory mechanisms act at three levels: 1) STATs level: reciprocal down-regulated STAT activation came from studies on genetically modified STAT-deficient cells [107]. The essential Th17 transcription activator STAT3 has been shown to be inhibited by STAT1, STAT2, STAT4 and STAT6 [107]; 2) cytokine level: signaling with the help of different STATs and other transcription factors, some cytokines inhibit Th17 development [108]. For example, IL-27, type I and II Interferons (IFN-α/β and IFN-γ respectively) tightly negatively regulate Th17 cell initiation and maintenance, as inferred from studies in mice [109-116] and humans [45, 117-121], mainly in a STAT1-dependant way. Th17 negative regulators also include IL-2 (via STAT-5) [122], IL-4 (via STAT6), IL-10 (via STAT3) and high dose TGF-β (via FOXP3) [100]; 3) Th cell level: Th1, Th2 and Treg show an antagonist effect intermediated by their respective signature cytokines mentioned earlier [108].

Different from Th1 and Th2, Th17 show a higher flexibility and heterogeneity concerning their initiation, maintenance and function. For example, Th17 differentiation could occur in the absence of TGF-β in mice, and only Th17 generated in this condition were shown to be pathogenic in an EAE model [123]. In some studies, Th17 proinflammatory effect could not be limited by blocking IL-17A or IL-17F [106]. These findings suggest that it may not be sufficient to define T-cell lineage based on a single cytokine and heterogeneity of effector T cells could be more complex than initially thought.

2.2.1.3 Th17-dependant skin and mucosal immunity to Candida

To date, there are six IL-17 family members [IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F], and five receptors (IL-17RA, IL-17RB/IL-25R, IL-17RC, IL-17RD/SEF and IL-17RE) (Table 2) [124]. Interleukin-17A and IL-17F are the most extensively studied. IL-17A/F producing CD4 T cells (Th17) specific for *C. albicans*, express on their surface the CCR4 and CCR6 receptors, which target them mainly to the skin and mucous membranes [125]. Epithelial cells express both IL-17RA, IL-17RC, IL-22R and IL-10R2 and thus can responses to Th17 cytokines mainly IL-17A, IL-17F (IL-17A/IL-17A and IL-17F/IL-17F homodimers or IL-17A/IL-17F heterodimers) and IL-22 [71, 96, 126]. Treatment of human bronchial lung epithelial (HBE) cells with IL-17 induces CXC chemokines such as IL-8 [71, 127], G-CSF[71], and antimicrobial proteins such as human β-defensin 2 (HBD2) [128]. IL-17 cytokines have also been shown to be important in regulating Th2 [129] and IgA [130] responses in the mucosa. IL-22 can activate STAT3 [131] and synergizes with IL-17 to increases the expression of human antimicrobial genes such as HBD2. A unique activity of IL-22, not shared with IL-17, is to increase the clonogenic potential of epithelial cells and accelerate wound repair [96]. More directly, treatment of skin keratinocytes with Th17 cytokines markedly increases anti-candidial activity *in vitro*, and this activity is lost when keratinocytes are tested in the presence of activated T cells from AD-HIES patients due to the absence of Th17 in these patients [132, 133]. Thus, Th17 cytokines are involved in mucocutaneous protection againt *C. albicans* (Figure 1) [56, 134-136]. Although Th17 have been suggested to be the main IL-17 cytokine producers, cellular sources of IL-17 include γδ T cells, innate NKT (iNKT) cells and innate lymphoid cells (iLCs) [137, 138]. Particularly in mucosal infections, the γδ T cells response can be the predominant source and their IL-17 production is critically regulated by both IL-23 and IL-1β [139, 140].

Table 2 IL-17 receptor and ligand families (adapted from [124]**)**

Receptor complex	Ligand(s)
IL-17RA/RC	IL-17A, IL-17F, IL-17A/F, viral IL-17a
IL-17RA/RB	IL-17E (a.k.a., IL-25)
IL-17RD (SEF)	Unknown
IL-17RA/RD	Unknown
IL-17RE	IL-17C
Unknown	IL-17D

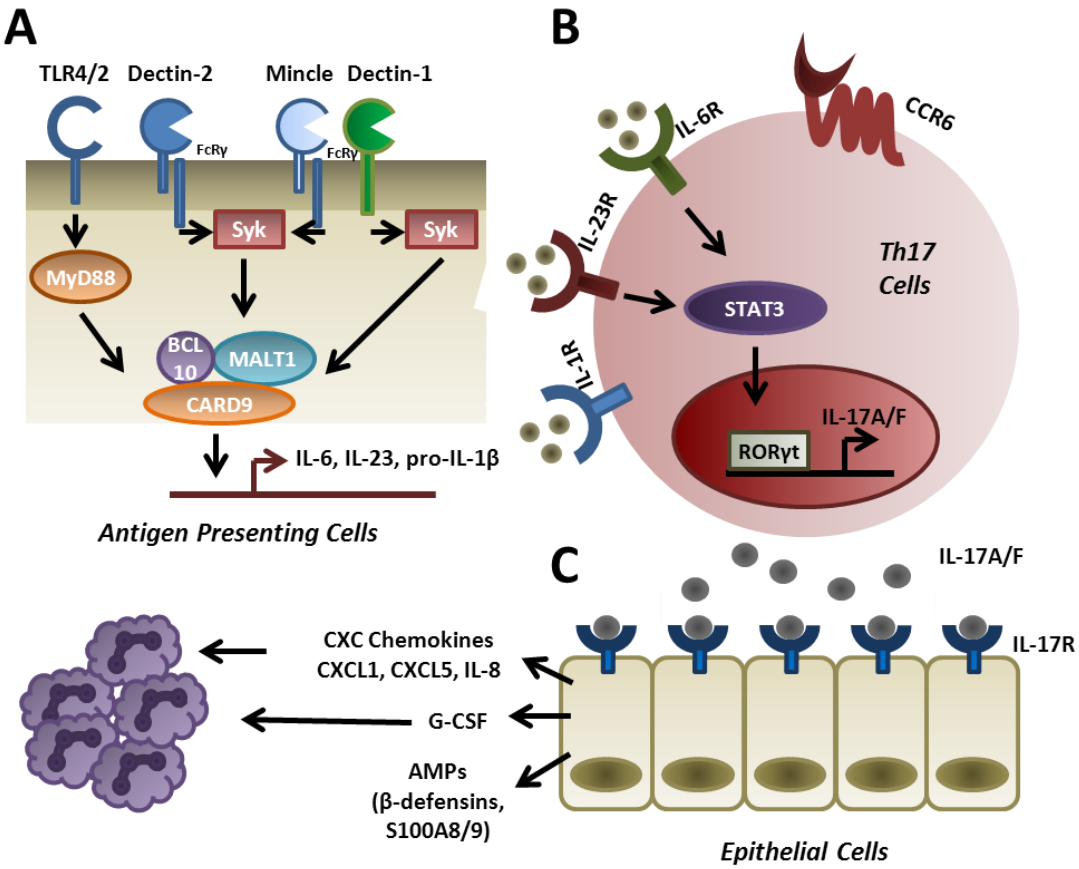

Figure 1: PRR and Th17-based immunity to *Candida albicans* (adapted from [88])
(A) PRRs including CLRs (Dectin-1, Dectin-2, MINCLE) and TLRs (TLR2, TLR4) respond to *Candida* PAMPs by inducing through Syk/CARD9/BcL10/MALT1 or MyD88, respectively, the NF-kB and MAPK pathways, leading to the secretion of pro-inflammatory cytokines such as IL-6, IL-23, and IL-1β.
(B) These cytokines bind to their receptors expressed on T cells, thereby inducing the differentiation of naïve T cells toward IL-17 producing T cells, in particular via the transcription factor STAT3, activated by IL-6, IL-23 (and IL-21) that in turn induces ROR-γt (ROR-γt for mice, its homologue RORC for human), leading to the transcription and the secretion of the IL-17 cytokines.
(C) IL-17A and IL-17F produced by Th17 cells bind to their receptors (IL-17RA/IL-17RC) expressed on various cells such as epithelial or mesenchymal cells to induce the expression of neutrophil attracting chemokines (IL-8, CXCL1, CXCL5) and activating/growth factors (G-CSF), as well as antimicrobial peptides (AMPs) such as defensins and S100 proteins [88]

2.2.2 Impaired IL-17 immunity in syndromic CMC

Considering that (i) IL-17 T cell plays an important role in immunity against *C. albicans* (based on studies in humans and mice), (ii) they are involved in the skin and mucous membranes protection, (iii) IL-6, IL-21, and IL-23 in particular, act via STAT3 to induce differentiation, proliferation and maintenance of IL-17 T cells, de Beaucoudrey *et al.* evaluated the presence of these cells in $STAT3^{+/-}$ patients with AD-HIES syndrome and syndromic CMC [23]. They have demonstrated in these patients, a significant decrease in the percentage of IL-17 T lymphocytes [55]. They have also observed a significant reduction in the proportion of these lymphocytes in patients deficient in IL-12p40 or IL-12Rβ1 with a complete lack of production or response to IL-12 and IL-23, respectively [55]. Although susceptible to mycobacterial infections or salmonella, approximately 25% of these patients develop moderate CMC [141]. These results therefore suggested that impaired IL-17 immunity could be at the origin of the syndromic CMC in these patients [21, 42, 43].

To further study the relation between impaired IL-17 immunity and CMC, our laboratory hypothesized that in APS-1 patient, CMC might result from autoimmunity against IL-17 cytokines. Indeed, these patients have numerous autoantibodies (auto-Abs), some directed against interferons IFN-α/-ω. However, these auto-Abs are probably not the cause of the associated CMC, as 65% of patients with thymomas have anti-IFN autoantibodies at diagnosis, and rare cases develop CMC [142. In addition, no CMC was reported in patients with various forms of STAT1 and TYK2 deficiency (unpublished data) and impaired responses to type I IFNs, or in patients with various forms of NEMO, UNC-93B, and TLR3 deficiencies and impaired production of type I IFNs {Al-Owain, 2010 #207]. In our laboratory, Puel *et al.* investigated the presence of auto-Abs against the IL-17s. In fact, along with another team, they found high titers of neutralizing auto-Abs against IL-17A, IL-17F, and/or IL-22, probably at the origin of the CMC occurrence in these patients [39-41]. These studies have contributed to the identification and characterization of the pathological mechanisms likely responsible for the CMC observed in these three syndromes, strongly suggesting that IL-17 T cells play a major role in mucocutaneous immunity against *C. albicans* in humans.

3. Results: CMCD and specific genetic defects of IL-17 immunity

3.1 Complete AR IL-17RA and partial AD IL-17F deficiency

According to literature, earlier studies had demonstrated the importance of IL-17 responses in host defense against mucosal candidiasis, both in mouse and human studies [88] and our laboratory results showed low IL-17 T cell proportions in PIDs with syndromic CMC [21, 42, 43], including AD-HIES syndrome [55] and AR APS-1 [41], as well as CARD9 deficient patients [27]. We therefore hypothesized that among CMCD patients, some may have a genetic defect affecting specifically IL-17-dependent immunity [21, 42, 43]. Puel *et al.* have undertaken the sequencing of candidate genes encoding IL-17s (including *IL-17A, IL-17F, IL-21, IL-22*, and *IL-26*) and their receptors in a cohort of CMCD patients recruited over the last years. Thus, in a child with CMCD (and skin infections with *S. aureus*) from a consanguineous family of Moroccan origin, we identified the first complete AR IL-17RA deficiency. The patient is carrying a homozygous mutation causing a premature stop codon located in the extracellular domain of IL-17RA (Q284X). This mutation was not found in any of public databases (1000 genomes, NCBI, Ensembl), nor in our own database of exomes (sequencing of the entire coding regions of the genome) sequenced in the laboratory (> 1000 today), nor among the 1052 control individuals from the Human Genome Diversity Project (HGDP) panel/ Center for the Study of Human Polymorphism (CEPH) panel, nor in 100 Moroccan controls sequenced, which excluded the variation being a polymorphism (i.e. frequent allele). I showed that this mutation abolished the expression of the IL-17RA protein at the surface of the patient's cells (fibroblasts and PBMCs), as well as their response to the homo- or hetero-dimers of IL-17A and IL-17F. Parents and siblings heterozygous for the mutation do not suffer from CMCD, demonstrating the recessive nature of the deficiency. Transfection of the patient's fibroblasts with a plasmid encoding the wild-type (WT), but not the mutant (Q284X) IL17RA or an empty plasmid, restored IL-17RA expression and the response to IL-17 cytokines in terms of IL-6 and Gro-α production. Thus, AR complete IL-17RA deficiency results in an abolished response to IL-17 cytokines and CMCD. At the same time, in a multiplex family with 5 CMCD individuals among 3 generations, we have identified the first partial AD

IL-17F deficiency, caused by a heterozygous missense mutation (S65L) in IL-17F. Again, it was not found in any of public or laboratory databases or CEPH control individuals, excluding a polymorphism. This mutation, located in the binding domain of the IL-17F to its receptor, is strongly hypomorphic (almost total loss of function) and dominant because it impairs the functionality of homo- (IL-17F/IL-17F) and hetero- (IL-17A/IL-17F) dimers containing the mutated protein, by blocking the binding of these complexes to their receptors [Publication #1]. This study has validated our hypothesis. It led to the discovery of the first two genetic etiologies of CMCD, showing that it is indeed a primary immunodeficiency. It confirmed the major role of IL-17s in mucocutaneous immunity against *C. albicans* in humans. However, unlike the situation observed in mice, these cytokines seemed redundant in the protection against the most common pathogens, since none of the reported patients had developed any severe infectious diseases besides CMCD. However, it is absolutely necessary to identify other patients with these deficiencies to draw definitive conclusion.

3.2 Heterozygous gain-of-function (GOF) *STAT1* mutations

3.2.1 Heterozygous *STAT1* GOF mutations are a major genetic etiology of CMCD

After the identification of the first two genetic etiologies of CMCD with AR IL-17RA and AD IL-17F deficiencies in one family each, using a candidate gene approach, mainly focusing on IL-17 immunity [Publication #1], we performed a "genome-wide" approach, by using whole-exome sequencing (WES), to identify novel morbid genes. We first performed WES for 6 CMCD patients. In order to analyze the WES data of the 6 CMCD patients, I first decided to focus on genes related to IL-17 immunity, as it is essential in mucocutaneous defense against *Candida*, even though the candidate gene strategy focused on IL-17 immunity (sequencing of *IL17A, IL17F, IL17RA IL17RC, IL22, IL22RA1, IL10RB,*) we used, let us to identify only 1 patient with AR IL-17RA deficiency in one family and five patients with AD IL-17F deficiency in another family. This suggested either that the gene (or genes) involved in IL-17 immunity and mutated in the rest of the CMCD cohort was (or were) not included in the candidate gene

investigation; or that there was (or were) other pathogenesis mechanism(s) underlying CMC than impaired IL-17 immunity. Considering the first hypothesis probable, I focused on IL-17 immunity and enlarged the candidate gene list. I did this in three steps: 1) I chose all the genes found mutated in PIDs with syndromic or isolated CMC: *STAT3, AIRE, CARD9, IL17F, IL17RA, IL12B, IL12RB1*; 2) I also included essential genes involved in IL-17 immunity according to the literature, such as *IL17A, RORC, SYK etc.* The candidate genes chosen in these first two steps are named as "IL-17 immunity key genes"; 3) I then added 20 interacting partners to each of these "IL-17 immunity key genes". The choice of these partners was done based on an online program "STRING", which collects known and predicted protein interactions including direct (physical) and indirect (functional) associations [143]. My idea was therefore to first check for these "IL-17 immunity key genes" in the 6 patients' exome data; then to check their interacting partners, and if no mutation could be found in any patient for this gene-list, then to increase the "candidate network genes". I called this strategy "candidate network" (Figure n° 2). The strategy was therefore to combine the online gene network database (STRING) and the whole-exome sequencing data. Besides the "candidate network" strategy, I also performed studies by following "hypothesis-generating" strategy, which means trying to identify variations in one gene common in two or more patients without proposing pathogenesis hypothesis. Combining these different approaches, I identified in four out of the six patients, three different *STAT1* heterozygous missense variations. *STAT1* was the only "candidate network gene" which displayed variations in more than four patients. These variations were not reported in any public dababase (1,000-genome, National Center for Biotechnology Information NCBI, Ensembl, and dbSNP) or in our own database (250 exomes at that time), excluding the variations being polymorphisms but instead rare events (mutations). We confirmed these mutations by Sanger sequencing and also excluded their presence in the 1,052 controls from 52 ethnic groups from the HGD/CEPH panels. Thus, these mutations were suggested to be probably CMCD-causing variants rather than irrelevant polymorphisms.

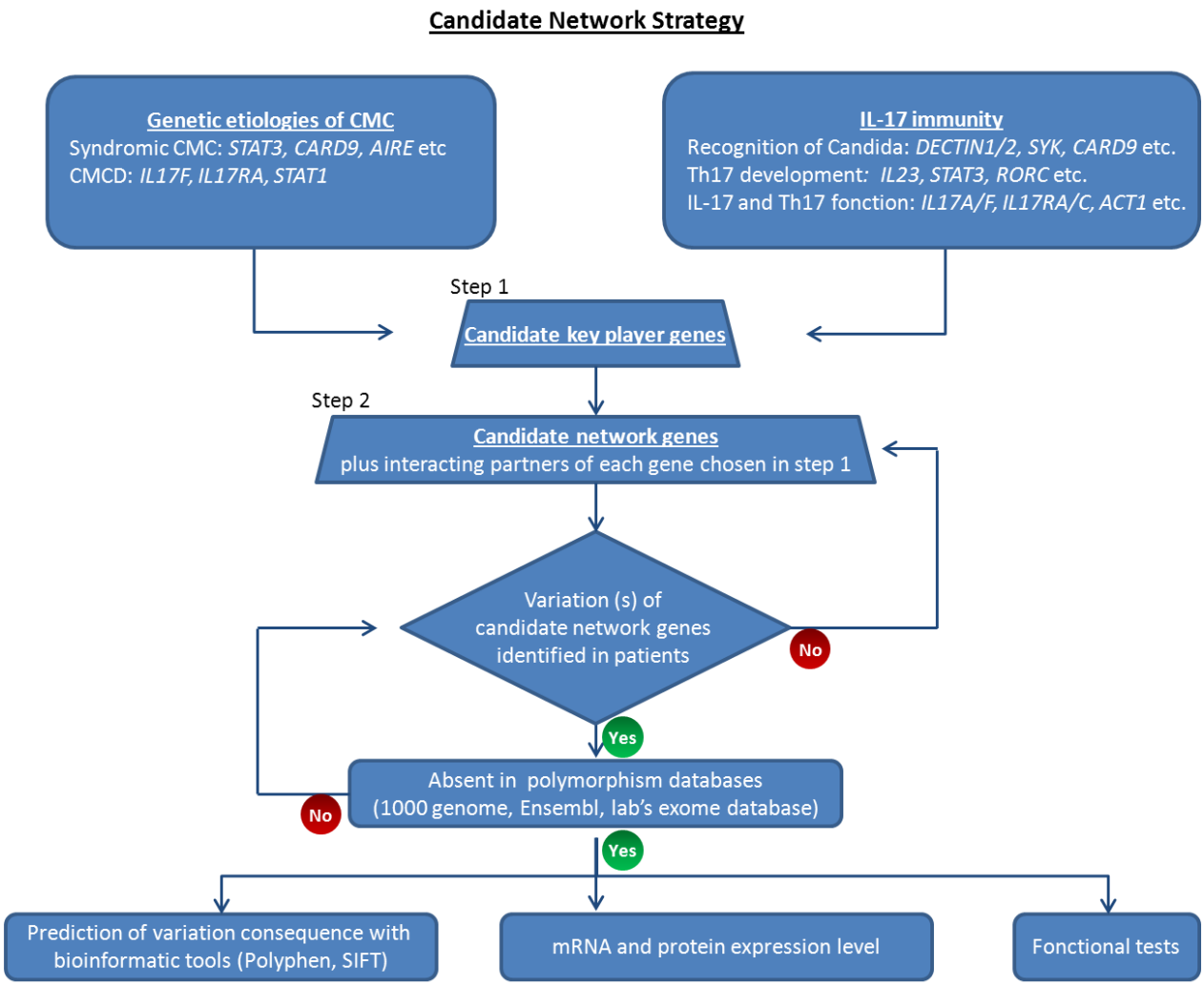

Figure 2 "Candidate gene network" strategy

1) the "candidate key player genes" were chosen according to both experimental data from mouse infection models and epidemiological studies in humans concerning IL-17 immunity and/or CMC pathogenesis; 2) "candidate gene network" list covered not only "key player genes" but also their interacting partners with the help of protein network database STRING; 3) verification of variations of candidate genes in patients and elimination of polymorphism. If no "non-polymorphsim" variation is identified in patient(s), the "candidate gene network" list is enlarged by adding more "key players" partners; 4) bioinformatics prediction and *in vitro* functional test of variation(s)' consequence.

3.2.2 Heterozygous *STAT1* variations identified in CMCD patients are gain-of-function mutations

We identified 139 CMCD patients from 74 families with 32 heterozygous missense mutations affecting the coiled-coil domain (CCD) (106 patients from 53 kindreds with 12 mutations) or the DNA binding domain (DBD) (33 patients from 21 kindreds with 20 mutations) of *STAT1* (Figure 3) [Publication #3], when we submitted the publication #3. To date, there are in total 55 heterozygous missense *STAT1* CMC mutations identified by us or other teams in 196 patients from 121 families (unpublished data). Their clinical penetrance appeared to be complete, as all CMCD patients from the kindreds tested were heterozygous, whereas none of these mutations was found at the heterozygous state in any of the healthy relatives sequenced. The mutations were not found in any public or in-house databases.

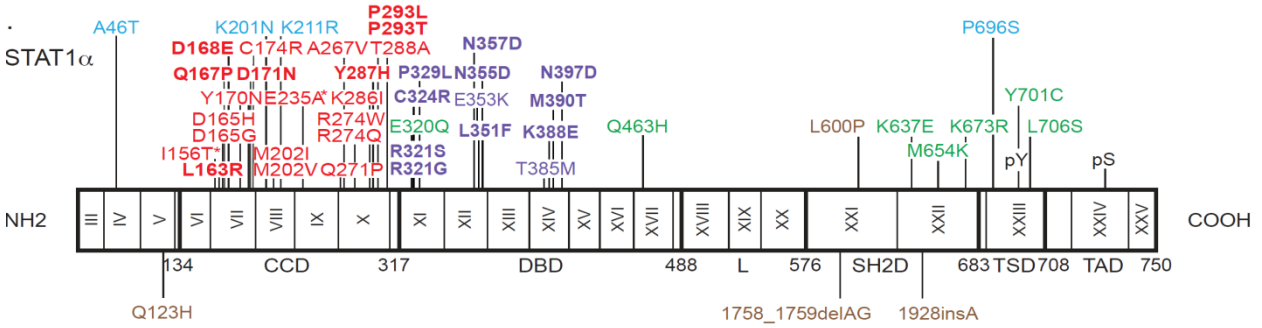

Figure 3 Heterozygous missense mutations affecting the CCD and DBD of STAT1 in kindreds with AD CMCD. The human STAT1 alpha isoform is shown, with its known pathogenic mutations. Coding exons are numbered with Roman numerals and delimited by a vertical bar. Regions corresponding to the coiled-coil domain (CCD), DNA-binding domain (DBD), linker domain (L), SH2 domain (SH2D), tail segment domain (TSD), and transactivator domain (TAD) are indicated, together with their amino-acid boundaries, and are delimited by bold lines. Tyr701 (pY) and Ser727 (pS) are indicated. Mutations in green are dominant and associated with partial STAT1 deficiency and Mendelian susceptibility to mycobacterial disease (MSMD). Mutations in brown are recessive and associated with complete STAT1 deficiency and intracellular bacterial and viral disease. Mutations in blue are recessive and associated with partial STAT1 deficiency and intracellular bacterial and/or viral disease. Mutations in red are dominant, located in the region encoding the CCD and associated with a gain of function of STAT1 and CMCD. Mutations in violet are dominant, located in the region encoding the DBD and associated with gain of function of STAT1 and CMCD..

The fact that heterozygous *STAT1* mutations were identified in more than half of the CMCD patients from our cohort (almost 300 patients) and absent in healthy controls, already gave a strong argument for the causality of these *STAT1* mutations in CMCD. However, the next question was what was the molecular impact of these *STAT1* alleles and how could they lead to the CMCD clinical phenotype?

Signal transducer and activator of transcription 1 (STAT1) belongs to a family of transcription factors comprising STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. STAT1 exists as two isoforms: STAT1α (91kDa) and STAT1β (84kDa) resulting from alternative splicing of the transcript. Both isoforms contain an N-terminal domain, a coiled-coil domain (CCD), a DNA-binding domain (DBD), and a SH2 domain. Only the STAT1α isoform possesses a transactivation domain (TAD) including two phosphorylated sites: tyrosine 701 and serine 727. Phosphorylated tyrosine 701 is required for STAT1 dimer formation and its transcription activity. STAT1 is an essential effector of IFNs including type I (IFN-α/β) and type II (IFN-γ), but also IFN-λ and IL-27. Following activation of the IFN-γ or IL-27 receptor, STAT1 is phosphorylated on tyrosine 701 and forms a homodimer. In response to IFN-α/β, phosphorylated STAT1 forms a heterotrimer with phosphorylated STAT2 and p48/IRF-9. Activated STAT1 homodimers or heterotrimers translocate into the nucleus, where they bind to specific consensus sequences: the GAS (gamma interferon activated sequence) or the ISRE (interferon stimulated response element) sequences, respectively. STAT1 homodimers (GAF: gamma interferon activating factor) bind to GAS DNA sequences via their N-terminal domain and stimulate the transcription of genes mainly involved in antibacterial immunity. STAT1/STAT2/p48 heterotrimers (ISGF3 complex) induce the transcription of genes involved in antiviral immunity via their binding to ISRE DNA sequences. STAT1 is also activated in response to other cytokines, including IL-6, IL-21 and IL-23; and in response to growth factors including EGF and PDGF [144]. STAT1 knockout mice respond very poorly to IFN-α/β, IFN-γ and IL-27. These mice are susceptible to viruses, bacteria and parasites [46]. However, when they are challenged with *C. albicans*, their response is similar to that of wild-type mice [145].

In human, biallelic or monoallelic mutations in *STAT1* had already been identified [46]. Human AR complete STAT1 deficiency leads to life-threatening intra-macrophagic bacterial and viral disease, and partial AR STAT1 deficiency presents a milder susceptibility to bacterial and viral diseases. AD STAT1 deficiency results in a rare syndrome characterized by infections with weakly pathogenic/virulent mycobacteria (Mendelian susceptibility to mycobacterial disease: MSMD syndrome). None of these STAT1 deficient patients had to our knowledge a susceptibility to fungal infection. Therefore, we made the hypothesis that the *STAT1* mutations identified in CMCD patients could be gain-of-function (GOF) instead of loss-of-function (LOF) as those previously identified, at least in response to IFNs and IL-27. To test this hypothesis, we functionally characterized the CMCD-causing STAT1 allele, R274Q, found in several kindreds. We compared it with a WT and an MSMD-causing LOF STAT1 allele (L706S). A higher STAT1 activity in response to IFNs and IL-27 was observed in STAT1-deficient U3C cells transfected with R274Q compared with WT or LOF alleles. Similarly, we also observed a higher STAT1 activity in Epstein-Barr (EBV) - transformed B cells from a patient heterozygous for the STAT1 R274Q allele compared with those from controls or LOF patients. These experiments demonstrated the GOF and the dominant nature of the CMCD-causing *STAT1* mutation [Publication #2].

3.2.3 GOF *STAT1* mutations lead to impaired IL-17 immunity in CMCD patients

The susceptibility to intracellular bacterial and/or viral infections in *STAT1* deficient patients is explained by impaired STAT1-dependent IFN immunity. In order to understand the pathological mechanism of *STAT1* GOF mutations in CMCD we asked two questions: 1) whether IL-17 immunity was impaired in these CMCD patients with STAT1 *GOF* alleles similarly to patients with syndromic CMC; 2) if so, whether *GOF* STAT1 mutations were the cause of the impaired IL-17 immunity.

Indeed, we highlighted an impaired development of IL-17-producing T cells, both *ex vivo* and after *in vitro* differentiation starting from CMCD patients' bulk leukocytes (Figure 4) [Publication #2], We also demonstrated that CMCD patients heterozygous for *STAT1* gain-of function alleles displayed poor IL-17-producing T-cell development from naïve CD4+ T cells after *in vitro* differentiation [Publication #3],

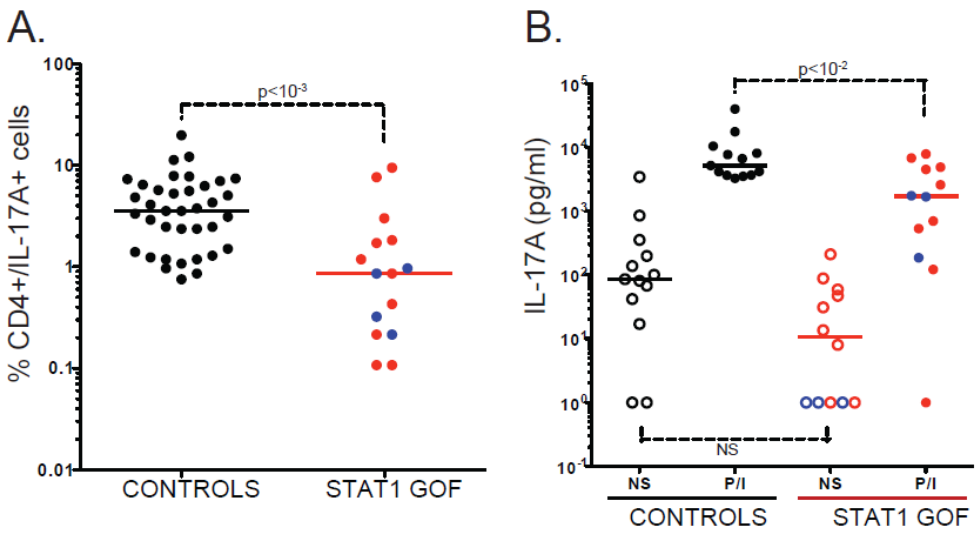

Figure 4 The differentiation of naïve CD45RA$^+$ CD4$^+$ T cells into IL-17- producing T cells *in vitro* is impaired in patients with AD CMCD and *STAT1* GOF mutations. (A-B) Proportion of IL-17A-producing T cells (A) and IL-17A secretion (B), after 12 days of naïve CD4$^+$ T cell differentiation in the presence of anti-CD3 and anti-CD28 antibodies, IL-1β and IL-23, as determined by flow cytometry and ELISA, respectively, after stimulation with PMA and ionomycin for 12 hours for flow cytometry analysis, and in the absence of stimulation (open symbols) or after 48 hours of stimulation (closed symbols) for ELISA analysis. Each symbol represents a value from a healthy control individual (black circles), a patient bearing a STAT1 gain-of-function (GOF) mutation affecting the CCD (red circles) or a patient bearing a STAT1 GOF mutation affecting the DBD (violet circles). Horizontal bars represent medians. The p-values for the nonparametric Wilcoxon tests are shown for comparisons of patients with STAT1 GOF mutations (STAT1 CCD: n = 11 and STAT1 DDB: n = 4) and controls (n = 37) (A) patients with STAT1 GOF mutations (STAT1 CCD: n = 9 and STAT1 DDB: n = 3) and controls (n = 13) (B).

3.2.3.1 Hypothesis I: major STAT1 activators (IFNs and IL-27) have a stronger inhibitory effect on IL-17 T cell differentiation in CMCD patients.

Both mouse [109-116] and human [45, 117-121] studies have shown that IFNs and IL-27 inhibit Th17 cell development in a STAT1 dependent manner in mice and humans. Thus, we hypothesized that CMCD in patients with GOF *STAT1* mutations could result from stronger STAT1 dependent inhibitory effect, downstream of IFNs and IL-27, on IL-17 T cell differentiation. To test this hypothesis, I first investigated IL-17 T cell differentiation starting from naïve CD4 T cells as an *in vitro* model to investigate whether the CCD and DBD STAT1 mutations impaired the development of IL-17 T cells. A combination of TGF-β

and IL-6 had been shown to be essential for the initial differentiation of IL-17 T cells in mice [146, 147], but the key cytokines required in humans remained less clearly defined (Sallusto et al., 2012). Various combinations of cytokines had been used for the differentiation of human IL-17 T cells: (1) TGF-β and IL-21; (2) IL-1β and IL-6; (3) IL-1β and IL-23 and (4) TGF-β and IL-23 [148-153]. I thus purified $CD45RA^+$ $CD4^+$ T cells by magnetic beads and cultured them in the presence of coated antibody (Ab) against CD3 and soluble Ab against CD28, together with individual cytokines or all possible combinations of TGF-β, IL-1β, IL-6, IL-21 and IL-23, in the presence of IL-2. I measured the proportion of IL-17A-expressing T cells and the secretion of IL-17A from days 5 to 12, by flow cytometry and ELISA, respectively. I obtained the most reproducible results within and between controls with a combination of IL-1β and IL-23 for 12 days (data not shown). IL-6 was not retained, as it increased inter-individual variability. In these conditions, I showed that patients heterozygous for CCD or DBD *STAT1* mutations had lower ($p < 10^{-3}$) proportions of IL-17A T cells and secreted smaller amounts of IL-17A ($p < 10^{-2}$) [Publication #3], Impaired IL-17 T-cell development in these patients was similar to that seen in patients with other conditions conferring CMC, including AD-HIES patients with heterozygous LOF *STAT3* mutations and AR MSMD patients with biallelic *IL12RB1* LOF mutations [Publication #3]. Collectively, these data demonstrate that the GOF *STAT1* mutations caused CMCD by impairing IL-17 T-cell immunity.

To further investigate the mechanisms by which CMCD-causing GOF *STAT1* alleles prevent the development of IL-17 T cells, in the culture conditions defined above, I added a combination of suboptimal doses of Th17 cell differentiation inhibitors (IFN-α2a, IFN-β1a, IFN-γ and IL-27). I observed a large decrease in the proportion of IL-17A T cells and in the secretion of IL-17A, in both the healthy controls and the STAT1 patients tested [Publication #3]. The effect was statistically significant when measured both by flow cytometry and ELISA, for CMCD patients ($p < 10^{-3}$), and for controls ($p < 5 \times 10^{-3}$ and $p < 10^{-2}$ respectively). Moreover, in these inhibitory conditions, the difference in terms of IL-17A T cell proportion and IL-17A production between controls and patients was more significant than in the absence of IFNs and IL-27 ($p < 10^{-3}$) [Publication #3]. At higher concentrations of IFN and IL-27, a

stronger inhibition was observed in the cells of controls and patients, with similar levels of inhibition in both [Publication #3]. These data suggested that the poor development of IL-17 T cells in patients heterozygous for *STAT1* alleles might result, at least in part, from enhanced IFN-α/β, IFN-γ and IL-27 responses via STAT1. I tested this hypothesis, by treating the cells with a combination of neutralizing Abs against IFN-α/βR2, IFN-γ and IL-27. These Abs rescued the development of IL-17 T cells carrying GOF *STAT1* mutations, whereas this effect was not detectable in healthy control IL-17 T cells [Publication #3]. Indeed, the effect of these Abs reached significance only in the patients' cells ($p < 10^{-3}$ by flow cytometry and $p < 5. \times 10^{-4}$ by ELISA). Moreover, in these conditions, the difference between the cells of the controls and those of the patients was abolished [Publication #3]. In these conditions, the proportion of $CD4^+$ IFN-γ^+ was slightly lower in the patients' cells, whereas the amounts of IFN-γ and IL-27 secreted were similar for controls and patients [Publication #3]. Overall, these experiments established that the poor development of IL-17 T cells in CMCD patients carrying GOF mutations affecting the CCD or DBD of STAT1 involves STAT1-dependent inhibition via IFN-α/β, IFN-γ and/or IL-27.

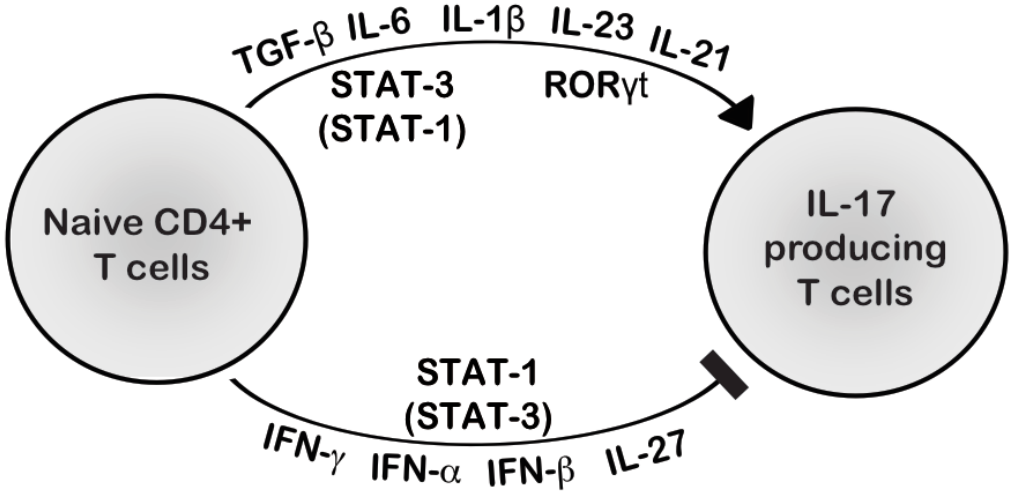

Figure 5: hypothesis I: major STAT1 activators (IFNs and IL-27) have a stronger inhibitory effect on IL-17 T cell differentiation in CMCD patients. Activating molecules, such as IL-23 and IL-21 (acting mostly through STAT3, and to a lesser extent, STAT1), IL-6, IL-1β, and TGF-β ,and inhibiting molecules, such as IFN-α/β, IFN-γ, and IL-27 (acting mostly through STAT1 and, to a lesser extent, STAT3) are represented.

3.2.3.2 Hypothesis II: higher STAT1 activity impacts on STAT3 activity and impairs IL-17 T cell development in CMCD patients

STAT1 and STAT3 have opposing roles in regulating survival/proliferation, inflammation [154]. They are both targets of cytokines and growth factors including types I Interferon and gp130 family cytokines such as IL-6. However, these mediators exert distinct and often opposing effects by activating STATs in specific patterns and duration. For example, IFN-α/β, IFN-γ and IL-27 predominantly active STAT1, in addition, activate STAT3, although to a lesser extent/more transiently. Cytokines such as IL-6, IL-21, IL-22 and IL-23 predominantly activate STAT3 and to a lesser extent STAT1. STAT1 and STAT3 can heterodimerize and bind to similar cognate sites at least *in vitro*, though in vivo their heterodimer function is not yet clear. STAT1 and STAT3 activation are reciprocally regulated and perturbation in their balanced expression or phosphorylation levels may re-direct cytokine/growth factor signals. For example, in the absence of STAT3, IL-6 triggers cellular responses super-imposable to those elicited by IFN-γ, correlating with prolonged STAT1 activation, in murine embryonic fibroblasts [155]. STAT1:STAT3 cross-regulation may act at different levels: 1) competition for common receptor docking sites. For example, in a study of alternative activation of STAT1 and STAT3 in response to interferon-gamma, the tyrosine 419 of the IFN-γ receptor subunit 1 (IFNGR1) was required to activate both STATs, suggesting that STAT1 and STAT3 could compete with each other for the same receptor phosphotyrosine motif [156]; 2) antagonist effect could be mediated by SOCS family. Distinct genes belonging to the SOCS family are induced as immediate early genes downstream of different STATs and are able to inhibit, by different mechanisms, STATs' phosphorylation in a classical negative-feedback loop [157]. For example, STAT3 activity can be prevented by SOCS1 which is induced in a STAT1-dependant way; 3) as there is a dynamic balance between STAT1/STAT1, STAT3/STAT3 homodimer and STAT1/STAT3 heterodimer formation, GOF *STAT1* mutations could trigger a shift from STAT3/STAT3 homodimers to STAT3/STAT1 and STAT1/STAT1 hetero- and homodimer formation, causing impaired STAT3/STAT3 homodimers' transcription activity. Considering the fact that IL-17 T cell development involves the

formation of STAT3 homodimers, their perturbation could lead to a defect in this development. Therefore, GOF *STAT1* mutations could impact on normal STAT3 activity, essential for Th17 development. However, in our study, STAT3 activation seems intact in terms of phosphorylation and homodimer formation in response to IL-6, IL-21 and IL-23. However, this hypothesis needs to be further investigated, as we have not yet studied the transcription of STAT3 target genes. In addition, STAT3 activity was only tested in patients' EBV-B cells. As STAT3 has distinction function in different cell types, its activity should be tested in other cells.

4. Conclusions

In conclusion, the work done by the laboratory on the dissection of the pathological mechanisms of syndromic CMC in AD-HIES and AR APS-1, strongly suggested an important role of IL-17 immunity in mucocutaneous protection against the fungus *C. albicans* [21, 23, 41-43, 55]. Based on these previous studies, we identified the first three genetic etiologies of CMCD: AR IL-17RA and AD IL-17F deficiencies [158] and gain-of-function mutations of *STAT1*, all associated with an impairment of IL-17 immunity [45, 46, 50] (Figure 6). Gain-of-function *STAT1* mutations currently represent the most frequent genetic cause of CMCD with a total of 94 patients reported in the literature since 2011 [45, 46, 50]. We have demonstrated that CMCD is a primary immunodeficiency associated with an impaired IL-17 immunity. Moreover, the fact that IL-17RA$^{-/-}$ or IL-17F$^{+/-}$ CMCD patients present increased susceptibility particularly to *C. albicans* and, to a lesser extent, to *S. aureus* cutaneous infections, but not to other pathogens demonstrated a major role of IL-17s in mucocutaneous Candidal and staphylococcal defense. In contrast to the results obtained in mice, IL-17s seem redundant for protection immunity against most other common pathogens. However more patients IL-17RA and IL-17 deficient are needed to be identified to draw definitive conclusions.

These studies also highlighted that distinct alleles of the same gene (*STAT1*) are capable of generating a susceptibility to different pathogens: complete or partial loss of function *STAT1* mutations are associated

with a lack of response to IFNs (IFN-α/β, IFN-γ, IFN-λ) and IL-27 and are associated with an increased susceptibility to intracellular bacterial and/or viral infections. In contrast, gain-of-function mutations, associated with increased responses to IFNs (IFN-α/β, IFN-γ, IFN-λ) and IL-27 result in impaired production of IL-17s and an increased susceptibility to CMCD. Finally, the increased responses to IFN-α/β could explain the thyroid autoimmune disease observed in some CMCD patients, such reactions are often reported as side effects of treatment with recombinant IFN-α or IFN-β [159, 160].

My work has contributed to validate the hypothesis that children with a susceptibility to a narrow spectrum of pathogen or even to a single pathogen may present a Mendelian genetic defect that specifically affects their immune response to certain infectious agents. It also allowed the inclusion of the susceptibility to localized and chronic *C. albicans* infection in the "unconventional" primary immunodeficiencies. It offered a better understanding of the role of IL-17s in mucocutaneous immunity against a common fungal disease (CMC), in natural conditions of infection in humans. Finally, these studies have important implications in the field of immunology with the description and characterization of the biological mechanisms involved in protective immunity specific for *C. albicans* and a better understanding of the pathophysiological mechanisms associated with increased susceptibility to fungal infections *in Natura* and in the medical field, with the possibility of molecular diagnostics, genetic counseling in case of positive diagnosis, better management care and prevention strategies in genetically predisposed individuals according to their defects. The identified immunological mechanisms can be targeted for the development of therapeutic and prophylactic treatments by restoring or strengthening impaired immunity (i.e. G-CSF for example).

Although we have demonstrated higher STAT1 activity in response to IL-17 inhibitors in CMCD patients' cells, and rescued at least partially impaired *in vitro* IL-17 producing T cells differentiation by blocking IL-17 inhibitors with neutralizing antibodies, the exact GOF STAT1 mutations impact on *in vivo* human IL-17 immunity mechanisms need to be further elucidated. We need to refine our *in vitro* IL-17 producing T cell differentiation from naïve CD4 T cells experiment protocol in order to reduce the high variability

within healthy controls or patients groups. At the same time, we should develop an *in vitro* test of Candida recognition by Antigen-presenting cell (APC) cell, and co-culture of APC and CD4 T cell at the presence of *Candida* to mimic *in vivo* Candida recognition and subsequent Th17 development.

For approximately half of the patients enrolled, no genetic defect has been identified yet. Exome sequencing is being performed for them. For some of the patients, we have already tested the proportion of *ex vivo* IL-17 cells and we are testing other *in vitro* IL-17 T cell differentiation conditions. Different exome analysis strategy can be adapted according to the patients' IL-17 T cell proportion profiles. Hypothesis-generating strategy will be more suitable for the patients without impaired IL-17 immunity. For the patients with already identified reduced proportion of IL-17 T cells, exome analysis should first focus on genes involved in IL-17 immunity. If, in co-culture *in vitro* experiments, we identify a defect in the recognition of *C. albicans* by APC cells from a CMCD patient, we will first check in this patient's exome data the genes encoding PPR and their downstream signaling pathway partners. "Candidate gene network" strategy can be improved in several ways: 1) the choice of IL-17 immunity key players and their interaction partners can be done in a more unbiased way with the help of bioinformatics tools; 2) It is laborious to check for the genotype of candidate genes one by one in exome data. It will be much more efficient to do it with the help of computer programs. The two suggestions require a high bioinformatic skill. A closer collaboration is needed between biological and computer scientists.

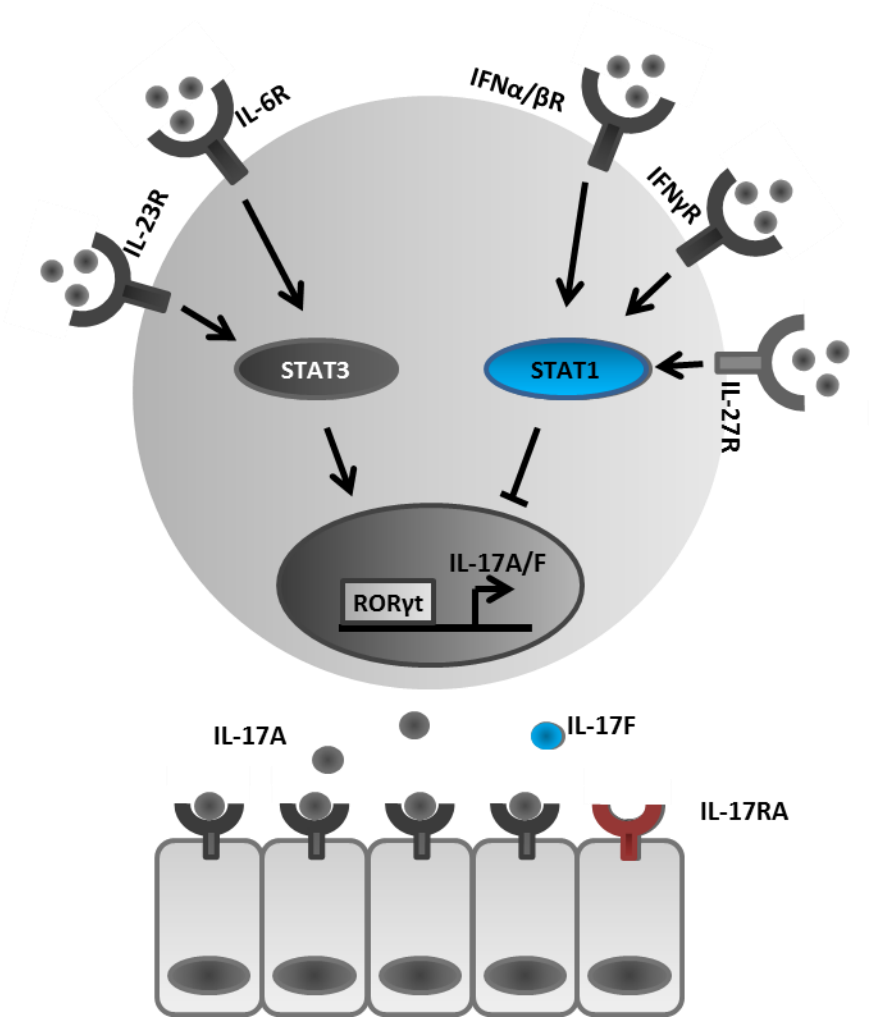

Figure 6. Three genetic etiologies of CMCD all leads to an impaired IL-17 immunity

CMCD-causing mutations in IL-17F (in blue) and IL-17RA (in red) impair IL-17 function and response, respectively. CMCD-causing STAT1 GOF mutations (also shown in blue) impair the development of IL-17-producing T cells. GOF *STAT1* mutations, associated with increased responses to IFNs (IFN-α/β, IFN-γ) and IL-27, are associated with an impaired production of IL-17s and an increased susceptibility to CMCD.

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Identification et caractérisation des bases génétiques moléculaires responsables de la prédisposition à la candidose cutanéo-muqueuse chronique chez l'homme

I. Introduction

L'exposition à un agent microbien est nécessaire pour le développement d'une maladie infectieuse mais n'est pas suffisante. Il est maintenant clairement établi que les facteurs génétiques de l'hôte jouent un rôle majeur dans sa susceptibilité à une maladie infectieuse [1, 2]. Dans certains cas, des défauts génétiques conférant une prédisposition Mendélienne à un nombre restreint, voire à un seul agent infectieux (1 gène, 1 infection) chez des individus par ailleurs sains ont été identifiés [3]. Dans ce contexte, je travaille sur le projet qui consiste en l'étude des bases génétiques et immunologiques de la susceptibilité accrue aux infections fongiques par le champignon *Candida albicans* dans le syndrome Mendélien de candidose cutanéo-muqueuse chronique isolée (CCMCi) « idiopathique ».

II. La CCMC: un rôle de l'immunité dépendante de l'IL-17

La CCMC est une maladie cliniquement complexe et hétérogène, principalement caractérisée par des infections sévères, persistantes ou récurrentes, souvent réfractaires aux traitements, de la peau, des ongles et des muqueuses par *C. albicans* [4-9]. *C. albicans* est un commensal des muqueuses dans la population générale. Cependant, des patients présentant une immunodéficience primaire (ex : des immunodéficiences sévères combinées) ou acquise (ex : syndrome d'immunodéficience acquise) avec des défauts du nombre des lymphocytes T souffrent fréquemment d'une CCMC [2, 7, 10-14]. Cependant, chez tous ces individus, la CCMC est l'une parmi de nombreuses autres infections opportunistes auxquelles ces patients sont vulnérables.

Au contraire, la CCMC est l'une des infections majeures dans le syndrome autosomique dominant hyper IgE (AD-HIES), causé par des mutations hétérozygotes de *STAT3*, un facteur de transcription situé en aval d'un nombre important de cytokines telles que l'IL-6, l'IL-21, l'IL-22, l'IL-23 et bien d'autres encore [15, 16]. La CCMC est également le phénotype infectieux majeur rapporté dans la seule famille publiée à ce jour avec un défaut complet autosomique récessif (AR) en CARD9, qui présente également d'autres infections fongiques telles qu'une dermatophytose et des infections invasives à *C. albicans* [17]. CARD9 une protéine adaptatrice impliquée dans la voie de signalisation de récepteurs (comme Dectin-1, Dectin-2, MINCLE, ou le Mannose

Récepteur MR) capables de reconnaître des composants majeurs de la paroi cellulaire de certains champignons, notamment *C. albicans* [7-9, 18-22].

La CCMC est également fréquemment retrouvée dans certains tableaux auto-immuns avec des atteintes endocrinologiques, comme dans le syndrome AR APS-1 (autoimmune polyendocrine syndrome type 1, aussi appelé APECED) [23], dû à des mutations dans *AIRE* [24], qui code un facteur impliqué dans la tolérance des lymphocytes T [25]. La CCMC, présente chez plus de 90% des patients APS-1, est en général l'une des premières manifestations cliniques et représente le seul phénotype infectieux rapporté chez ces patients [7-9, 26-29] [Publication #2]. Dans la suite de cette synthèse, la CMCC associée à ces conditions (syndromes AD-HIES, AR APS-1 ou le déficit AR en CARD9) sera référencée comme CCMC syndromique.

Enfin, la CCMC peut exister sous forme isolée (CCMCi) chez des individus par ailleurs sains, sans autre tableau clinique sévère associé, infectieux ou auto-immun [4-7]. Cependant, la CCMCi est associée à un risque accru de développer des carcinomes cellulaires squameux buccaux ou œsophagiens ou à des anévrismes cérébraux dont l'origine reste inconnue [7][Publication #2]. Il s'agit d'une maladie rare, qui apparaît le plus souvent tôt dans l'enfance et dont la prévalence est estimée à environ 1/100.000 individus.

Les premiers cas de CCMCi ont été décrits au début des années 1960 et, dès le début des années 1970, des formes familiales, avec une ségrégation principalement autosomique dominante (AD) ou plus rarement autosomique récessive (AR) ont été rapportées [4, 7]. Depuis, de nombreux cas sporadiques et familiaux ont été décrits [7], suggérant que la CCMCi pourrait résulter de défauts génétiques Mendéliens, au moins pour certains patients [7]. Cependant, aucune étiologie génétique et aucun phénotype immunologique robuste n'ont émergé, jusqu'à très récemment, grâce notamment à l'identification et la caractérisation des bases génétiques et des mécanismes physiopathologiques d'immunodéficiences primaires avec une CCMC syndromique [7-9, 27-33] [Publication #1, 2].

II.1 ***La CCMC syndromique : un rôle de l'immunité dépendante de l'IL-17 ?***

Au cours de ces dernières années, le développement de modèles murins de CMC (essentiellement au niveau oro-pharyngien) [34], la caractérisation du rôle des cytokines « IL-17s » (IL-17A, IL-17F et IL-22) [35, 36] et enfin la caractérisation moléculaire d'immunodéficiences primaires avec

une CCMC syndromique [7-9, 27-30, 33, 37][Publication #2] ont largement contribué à donner un nouvel essor à la recherche des bases génétiques de la prédisposition à la CCMCi.

Considérant que (i) les lymphocytes T IL-17 jouent un rôle important dans l'immunité envers *C. albicans* (d'après des études réalisées chez l'homme et chez la souris), (ii) qu'ils sont impliqués dans la protection de la peau et des muqueuses, (iii) que l'IL-6, l'lL-21, et l'IL-23, en particulier, agissent via STAT3 pour induire la différentiation, la prolifération et la maintenance des lymphocytes T IL-17, des études réalisées chez des patients $STAT3^{+/-}$ présentant le syndrome AD-HIES et une CCMC syndromique [30] ont mis en évidence une réduction très significative du pourcentage des lymphocytes T IL-17 chez ces patients [7-9, 33]. Une réduction significative de la proportion de ces lymphocytes T IL-17 a également été observée chez des patients déficients en IL-12p40 ou IL-12Rβ1 avec un défaut complet de production ou de réponse à l'IL-12 et l'IL-23 [7-9, 33]. Environ 25% de ces patients développent une CCMC modérée [38, 39]. Enfin, une réduction de la proportion des lymphocytes T IL-17 chez les patients déficients en $CARD9^{-/-}$ a également été rapportée [17]. Enfin, notre laboratoire a identifié la présence de titres élevés d'auto-anticorps IgG neutralisants dirigés contre l'IL-17Al'IL-17F et/ou l'IL-22, probablement à l'origine de la survenue de la CCMC chez les patients APS-1 [7-9, 26, 27, 32] [Publication #2]. Ces études ont donc contribué à l'identification et la caractérisation des mécanismes physiopathologiques probablement responsables de la CCMC observée dans ces syndromes, suggérant très fortement que l'immunité dépendante de l'IL-17 jouait un rôle majeur dans la défense mucocutanée vis-à-vis de *C. albicans* chez l'homme [7-9].

II.2 *La CCMC isolée et les défauts génétiques spécifiques de l'immunité IL-17*

II.2.1 Défauts complet AR en IL-17RA et partiel AD en IL-17F

Basés sur les données de la littérature et nos résultats récents, nous avons émis l'hypothèse que parmi les patients atteints de CCMCi, certains pourraient présenter un défaut génétique affectant spécifiquement l'immunité IL-17-dépendante [7-9]. Sur la cohorte recrutée, nous avons donc entrepris le séquençage des gènes candidats codant les IL-17s (notamment IL-17A, IL-17F, IL-21, IL-22, IL-26) et leurs récepteurs. Ainsi, chez un enfant présentant une CCMCi (avec des infections cutanées par *S. aureus*), issu d'une famille consanguine d'origine Marocaine, nous avons identifié le premier défaut complet AR en IL-17RA. Le patient est en effet porteur d'une mutation homozygote à l'origine d'un codon stop prématuré situé dans le domaine extracellulaire

de l'IL-17RA (Q284X). Cette mutation abolit l'expression de la protéine à la surface des cellules du patient, ainsi que leur réponse aux homo- ou hétéro-dimères d'IL-17A et d'IL-17F. Les parents et les frères et soeurs, hétérozygotes pour la mutation, ne souffrent pas de CCMCi, démontrant le caractère récessif du défaut [7, 8][Publication #1]. Dans le même temps, dans une famille multiplexe avec 5 individus atteints de CCMCi sur 3 générations, nous avons identifié le premier défaut partiel AD en IL-17F, provoqué par une mutation hétérozygote faux-sens (S65L) dans l'*IL17F*. Cette mutation, située dans le domaine de liaison de l'IL-17F à son récepteur, est très hypomorphe (perte presque totale de la fonction) et dominante car elle détériore la fonction des homo-(IL-17F/IL-17F) et des hétéro-dimères (IL-17A/IL-17F) qui contiennent la protéine mutée, en bloquant la liaison de ces complexes à leurs récepteurs [7, 8]. Cette étude a donc validé notre hypothèse de travail. Elle a permis la découverte des deux premières étiologies génétiques de la CCMCi, démontrant ainsi qu'il s'agit en effet d'une immunodéficience primaire. Elle a confirmé le rôle majeur des IL-17s dans l'immunité mucocutanée vis-à-vis de *C. albicans*, et dans une moindre mesure vis-à-vis de *S. aureus* chez l'Homme.

II.2.2 Mutations dominantes gain-de-fonction de STAT1

En parallèle, nous avons utilisé une seconde approche, globale, génératrice d'hypothèses, par séquençage complet des régions codantes du génome par whole exome sequencing (WES) [40-42]. Nous avons réalisé un premier WES sur 6 patients : un cas sporadique et 5 patients issus de 5 familles multiplexes présentant une forme AD de CCMCi. Pour 4 d'entre eux, j'ai identifié des mutations hétérozygotes faux-sens situées dans le domaine coiled-coil (CCD) du facteur de transcription STAT1. Nous avons confirmé ces mutations par séquençage classique (méthode de Sanger) et avons séquencé cette région de *STAT1* (exons 6 à 10) chez 106 autres patients, dont 57 cas sporadiques et 49 issus de 22 familles multiplexes avec une AD CCMCi. Au total, nous avons identifié 12 mutations hétérozygotes dans le CCD de *STAT1* chez 47 patients appartenant à 20 familles [7, 8, 28, 31]. Aucune de ces mutations n'a été trouvée dans les banques publiques (NCBI, Ensembl, dbSNP, 1000 genomes). Elles étaient également absentes des 1052 contrôles du CEPH, suggérant qu'elles sont des allèles rares, à l'origine de la CCMCi plutôt que des polymorphismes non pertinents. La ségrégation intra-familiale est cohérente avec un trait AD et une pénétrance complète, car tous les patients testés sont porteurs de la mutation à l'état hétérozygote, alors que les parents, sains, sont porteurs à l'état homozygote de l'allèle sauvage.

Cette découverte était particulièrement surprenante puisque des mutations mono- ou bi-alléliques de *STAT1*, nulles ou hypomorphes, avaient déjà été identifiées et démontrées comme conférant une susceptibilité accrue à des infections par des bactéries intracellulaires (en particulier des mycobactéries) et/ou des infections virales [28]. Ceci est expliqué par le fait que STAT1 est un facteur de transcription activé notamment par l'IFN-γ et l'IL-27 qui induisent la transcription de nombreux gènes impliqués dans l'immunité anti-mycobactérienne, et par les IFN-α/β et IFN-λ, qui induisent la transcription de gènes impliqués dans l'immunité anti-virale [28]. Par ailleurs, aucun de ces patients n'avait été rapporté, à notre connaissance, comme présentant une susceptibilité accrue à la CCMC.

Nous avons résolu cette énigme en démontrant que les mutations de STAT1 responsables de la CCMCi, contrairement à celles identifiées précédemment, ne résultent pas en une perte de fonction mais au contraire en un gain de fonction de STAT1, qui se traduit par une phosphorylation accrue après activation par les IFNs ou l'IL-27, qui résulte d'un défaut de déphosphorylation de STAT1 dans le noya[Publication #2]. Nous avons ainsi montré que ces mutations engendrent un accroissement des réponses cellulaires STAT1-dépendantes aux cytokines telles que les IFN-γ, IFN-α/β, IFN-λ et l'IL-27, qui sont des répresseurs de la différentiation des lymphocytes T IL-17[7, 8, 27, 29]. En effet, j'ai mis en évidence des proportions significativement réduites de lymphocytes T producteurs d'IL-17s chez ces patients, rendant compte de la survenue de leur CCMCi [7, 8, 27, 29] [Publication #2,3]. Ce phénotype résulte de l'effet inhibiteur plus fort des STAT1-dépendantes Th17 répresseurs (IFN-γ, IFN-α/β, IFN-λ et l'IL-27) [Publication #3].

III. Conclusion

En conclusion, les travaux réalisés sur la dissection des mécanismes physiopathologiques de la CCMC syndromique, dans les syndromes AD-HIES, AR APS-1 et le déficit en CARD9 ont fortement suggéré un rôle essentiel de l'immunité dépendante de l'IL-17 dans la protection cutanéo-muqueuse vis-à-vis du champignon *C. albicans* [27, 30, 32, 33]. Nous avons ainsi démontré que la CCMC isolée est une immunodéficience primaire, associée à un défaut de l'immunité réalisée par les IL-17s, avec l'identification, en 2011, des trois premières étiologies génétiques de la CCMCi, avec les défauts AR en IL-17RA, AD en IL-17F et des mutations gain-de-fonction de *STAT1* [7-9, 28, 31][Publication #1 , 2]. Des mutations gain-de-fonction de *STAT1*

représentent à ce jour la cause génétique la plus fréquente de la CCMCi avec au total 94 patients rapportés dans la littérature depuis 2011 [7, 8, 28, 31, 42-45][Publication #2].

Enfin, ces travaux ont des implications majeures dans le domaine immunologique avec la description et la caractérisation des mécanismes biologiques impliqués dans l'immunité protectrice spécifique de *C. albicans* et une meilleure compréhension des mécanismes physiopathologiques associés à une susceptibilité accrue aux infections fongiques, dans des conditions naturelles d'infection ; et dans le domaine médical, avec la possibilité de diagnostics moléculaires, un conseil génétique en cas de diagnostic positif, une meilleure prise en charge et des stratégies de prévention chez les individus génétiquement prédisposés en fonction de leur défaut. Les mécanismes immunologiques identifiés pourront être la cible privilégiée pour le développement de traitements thérapeutiques et prophylactiques visant à les restaurer ou les renforcer (i.e. G-CSF).

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ANNEX

Publication 1

Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity

Puel A*, Cypowyj S*, Bustamante J, Wright JF, **Liu L**, Lim HK, Migaud M, Israel L, Chrabieh M, Audry M, Gumbleton M, Toulon A, Bodemer C, El-Baghdadi J, Whitters M, Paradis T, Brooks J, Collins M, Wolfman NM, Al-Muhsen S, Galicchio M, Abel L, Picard C, Casanova JL..

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Chronic Mucocutaneous Candidiasis in Humans with Inborn Errors of Interleukin-17 Immunity

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Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1200970/DC1
Materials and Methods
Figs. A1 to E12
Tables SA1 to SE24
References and Notes

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Chronic Mucocutaneous Candidiasis in Humans with Inborn Errors of Interleukin-17 Immunity

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Chronic mucocutaneous candidiasis disease (CMCD) is characterized by recurrent or persistent infections of the skin, nails, and oral and genital mucosae caused by *Candida albicans* and, to a lesser extent, *Staphylococcus aureus*, in patients with no other infectious or autoimmune manifestations. We report two genetic etiologies of CMCD: autosomal recessive deficiency in the cytokine receptor, interleukin-17 receptor A (IL-17RA), and autosomal dominant deficiency of the cytokine interleukin-17F (IL-17F). IL-17RA deficiency is complete, abolishing cellular responses to IL-17A and IL-17F homo- and heterodimers. By contrast, IL-17F deficiency is partial, with mutant IL-17F–containing homo- and heterodimers displaying impaired, but not abolished, activity. These experiments of nature indicate that human IL-17A and IL-17F are essential for mucocutaneous immunity against *C. albicans*, but otherwise largely redundant.

Chronic mucocutaneous candidiasis (CMC) is characterized by infections of the skin, nails, and oral and genital mucosae with *Candida albicans*, which is commensal in healthy individuals (*1*). In patients with inherited or acquired T cell immunodeficiencies, CMC is associated with various infectious diseases (*1*). In patients with STAT3 deficiency and a lack of interleukin-17A (IL-17A)– and IL-22–producing T cells (*2*–*5*), CMC is associated with severe cutaneous and pulmonary staphylococcal infections (*1*). In some patients with IL-12p40 or interleukin-12 receptor β1 (IL-12Rβ1) deficiency and mycobacterial disease (*2*) and in a family with caspase recruitment domain 9 (CARD9) deficiency with systemic candidiasis and peripheral dermatophytosis (*6*), CMC and low proportions of IL-17A–producing T cells were also documented. Finally, CMC is the only infection of patients with autoimmune regulator (AIRE) deficiency, who have neutralizing autoantibodies against IL-17A, IL-17F, and/or IL-22 (*7*, *8*). These data suggest that human IL-17A, IL-17F, and/or IL-22 are involved in mucocutaneous immunity to *C. albicans* (*1*). CMC disease (CMCD), the molecular and cellular basis of which is unknown, consists of CMC in the absence of other overt infectious or autoimmune signs (*1*). CMCD was initially thought to be benign, until squamous cell carcinoma (*9*) and cerebral aneurysms (*10*) were reported. First described in 1967 in sporadic cases (*11*), familial CMC segregating as autosomal dominant (AD) (*12*) and autosomal recessive (AR) traits (*13*) was soon reported. We thus searched for the genetic basis of CMCD, testing the hypothesis that CMCD may be caused by inborn errors of IL-17A, IL-17F, or IL-22 immunity (*1*, *14*).

Autosomal recessive IL-17RA deficiency. We first investigated a French child born to first-cousin parents of Moroccan descent (Fig. 1A) [report S1 (*15*)]. He presented with *C. albicans* dermatitis during the neonatal period and dis-

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played *Staphylococcus aureus* dermatitis at 5 months of age. Known causes of CMC were excluded clinically and genetically, and the lack of any phenotype other than CMC led to a diagnosis of AR CMCD. We sequenced the candidate genes encoding IL-22, IL-22RA1, IL-10RB, IL-17A, IL-17F, IL-17RA, and IL-17RC (*16*–*18*). IL-22 binds as a monomer to its receptor, composed of IL-22RA1 and IL-10RB, whereas IL-17A and IL-17F can form homo- or heterodimers that signal via a receptor comprising IL-17RA and IL-17RC chains. The child was found to be homozygous for the c.850C>T nonsense mutation (c.850C>T/c.850C>T), which replaces the glutamine codon in position 284 with a stop codon (Q284X/Q284X) in the *IL17RA* gene (*19*) (Fig. 1B). This premature stop codon is located in the part of the gene encoding the extracellular domain of IL-17RA, upstream from the transmembrane domain sequence (Fig. 1C). No mutations were found elsewhere in *IL17RA* or in any of the other six genes sequenced. The parents and siblings of this child are healthy and heterozygous for the mutant allele, consistent with AR inheritance for this trait. The mutant allele was not found in 1065 healthy controls from 52 ethnic groups from the Centre d'Etude du Polymorphisme Humain–Human Genome Diversity Cell Line Panel CEPH-HGDP, 100 French controls, and 70 Moroccan controls of Berber descent, which ruled out an irrelevant polymorphism and suggested that the mutation may define a rare AR CMCD-causing allele.

The IL-17RA protein was not detected on the surface of fibroblasts, peripheral blood mononuclear cells (PBMCs), or, more specifically, CD4+ T cells, CD8+ T cells, and monocytes from the patient, as shown by flow cytometry with two specific antibodies against the extracellular domain (Fig. 2A and fig. S1). The absence of IL-17RA had no impact on the expression of IL-17RC, which was normal on the patient's monocytes (the only leukocyte subset expressing IL-17RC in controls) and fibroblasts (figs. S1 and S2). Likewise, IL-22RA1 was normally expressed on the patient's fibroblasts (fig. S2). The patient also had a normal proportion of circulating IL-17A– and IL-22–producing T cells (fig. S3). We investigated whether the lack of IL-17RA expression had any functional consequences for the response to IL-17 cytokines, by testing the responses of the patient's fibroblasts to various concentrations of recombinant IL-17A and IL-17F homodimers and to IL-17A–IL-17F heterodimers (*17*, *18*). Like nuclear factor-κB essential modulator (NEMO)–deficient fibroblasts, which have impaired NF-κB activity, and unlike fibroblasts from a healthy control, the patient's fibroblasts did not respond to any of the three IL-17 cytokines, in terms of IL-6 and growth-regulated oncogene-α (GRO-α) induction (*20*), as assessed by enzyme-linked immunosorbent assay (ELISA) on supernatants (Fig. 2, B and C). Moreover, the patient's PBMCs did not respond above baseline to IL-17A or IL-17F for any of the cytokines tested (fig. S4A). Transfection of the patient's fibroblasts with wild-type (WT) *IL17RA*, but not with a mock vector, restored IL-17RA expression and the response to IL-17 cytokines (Fig. 2, D to F). By contrast, IL-6 production by NEMO-deficient cells was not rescued by transfection with *IL17RA* (fig. S4B). Thus, the patient with CMCD that we studied displayed AR, complete IL-17RA deficiency, and a lack of cellular responses to at least three IL-17 cytokine dimers—IL-17A, IL-17F, and IL-17A–IL-17F—in fibroblasts and leukocytes.

Autosomal dominant IL-17F deficiency. We then investigated a multiplex family from Argentina, with AD inheritance of CMCD (Fig. 3A) [report S2 (*15*)]. The *IL22*, *IL22RA*, *IL10RB*, *IL17RA*, *IL17RC*, and *IL17A* genes contained no mutations, but a heterozygous missense mutation was found in the *IL17F* gene of the index case. This mutation, c.284C>T, replaced the serine residue in position 65 of the mature protein with a leucine residue (S65L) (Fig. 3, B and C). The Ser^{65} residue is conserved across mammalian species (fig. S5). Moreover, the sequencing of 1074 control individuals from the CEPH-HGD panel ruled out the possibility that this mutation was an irrelevant polymorphism. Computational analysis showed that Ser^{65} lies in the cavity of the protein, which is thought to be involved in cytokine-to-receptor binding (Fig. 3C) (*21*). No other *IL17F* variations were found in the index case, including the *IL17F* g.7488T>C (rs763780) polymorphism, in which an arginine residue replaced a histidine in position 161 of the protein (H161R), a mutation previously thought to be loss-of-function (*22*). By contrast, we found that the H161R allele encoded an IL-17F protein able to stimulate murine lung epithelial cells (MLEs) (fig. S6). Heterozygosity for the S65L allele was found in all tested members of the kindred with CMCD; we were unable to genotype the fifth patient (III.1 in Fig. 3A), who died at 6 years of age from complications of the disease. The mutant allele was found in only two apparently healthy family members, aged 9 months (III.3 in Fig. 3A) and 21 years (II.8 in Fig. 3A), which suggested incomplete clinical penetrance. We did not detect IL-17F–expressing T cells in controls by flow cytometry, but the patients tested displayed normal proportions of IL-17A– and IL-22–expressing T cells, and their PBMCs secreted normal amounts of cytokines, as measured by Bioplex (fig. S7, A and B).

We investigated the possible deleterious effects of the S65L mutation by producing the mutant IL-17F protein in human embryonic kidney (HEK) 293 cells. The mutation did not seem to affect production of the monomeric protein or the formation of IL-17F homodimers (mutant-mutant and wild-type–mutant) or heterodimers with IL-17A (fig. S8). The mutant-containing dimers seemed to bind normally to homodimeric IL-17 receptors (IL-17RA and IL-17RC), as shown by surface plasmon resonance (table S1 and fig. S9). However, the mutant proteins did not bind IL-17RA on fibroblasts, as shown by flow cytometry, with IL-17RA–deficient cells as controls (confirming that their lack of IL-17RA expression prevented cytokine binding) (figs. S10 and S11). Accordingly, when control fibroblasts (Fig. 4, A and B) and keratinocytes (fig. S12, A and B) were stimulated with mutant S65L IL-17F homodimers, they displayed much weaker IL-6 and GRO-α induction than observed with WT IL-17F homodimers (IL-17WT), IL-17A homodimers, or IL-17A–IL-17FWT heterodimers (*20*). Moreover, control PBMCs showed impaired induction of several cytokines when stimulated with S65L IL-17F homodimers compared with

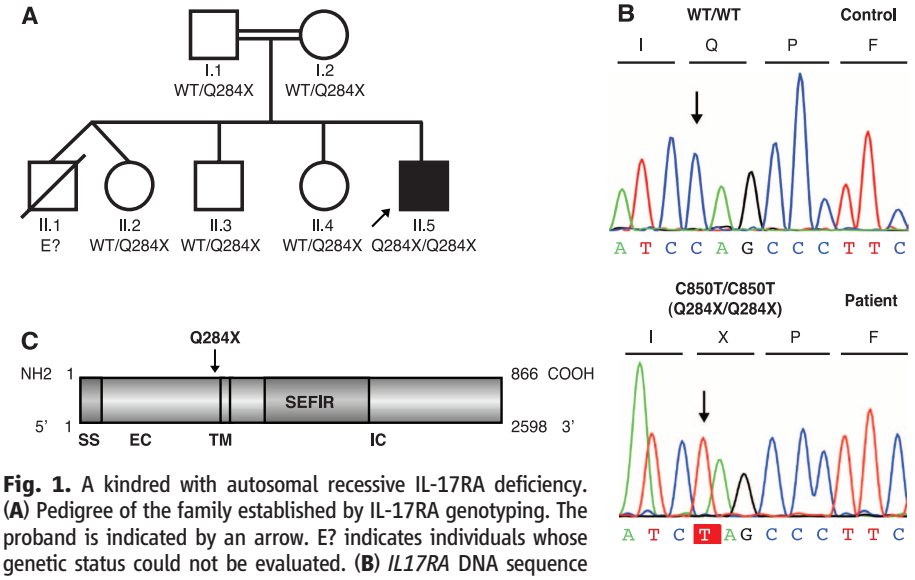

Fig. 1. A kindred with autosomal recessive IL-17RA deficiency. (**A**) Pedigree of the family established by IL-17RA genotyping. The proband is indicated by an arrow. E? indicates individuals whose genetic status could not be evaluated. (**B**) *IL17RA* DNA sequence electrophoregrams for a control and the patient. (**C**) Schematic diagram of the IL-17RA protein with the signal sequence (SS), extracellular (EC), transmembrane (TM), intracellular (IC), and SEFIR (expression similar to fibroblast growth factor–IL-17R) domains and the position within the extracellular domain affected by the mutation.

WT IL-17F homodimers (fig. S12C). These data suggest that the *IL17F* S65L allele is severely hypomorphic (Fig. 4, A and B, and fig. S12, A and B). Furthermore, when the S65L mutant IL-17F formed a heterodimer with either IL-17FWT or IL-17A, the induction of IL-6 and GRO-α was severely impaired in control fibroblasts (Fig. 4, A and B) and keratinocytes (fig. S12, A and B), which indicated a dominant-negative effect of this allele. Finally, as predicted by the lack of binding of mutant cytokine dimers to their receptor (fig. S11), these dimers did not compete with WT dimers (fig. S13, A to D). Thus, the AD CMCD in this kindred results from a hypomorphic, dominant-negative *IL17F* allele, which impairs the receptor binding and bioactivity of both IL-17F homodimers and IL-17A–IL-17F heterodimers.

Concluding remarks. IL-17RA and IL-17F deficiencies underlying mucocutaneous disease caused by *C. albicans* and, to a lesser extent, *S. aureus* are consistent with the mouse model (*23*). IL-17RA– and IL-17RC–deficient mice were more susceptible to oropharyngeal candidiasis (*24*, *25*) and IL-17RA–deficient mice to cutaneous staphylococcal disease (*26*). IL-17A–deficient mice also display impaired clearance of *C. albicans* skin infection (*27*). IL-17F–deficient mice have not yet been tested, but IL-23–deficient mice with impaired expression of IL-17A and IL-17F are also vulnerable (*27*). IL-17A or IL-17F alone are not required for peripheral immunity to *S. aureus*, but mice deficient for both IL-17A and IL-17F display an impaired peripheral immunity to *S. aureus* (*28*). Somewhat at odds with our observations, IL-17A is also required for systemic immunity to *C. albicans* (*29*) and *S. aureus* (*30*). Moreover, mice with IL-17RA, IL-17RC, IL-17A, or IL-17F deficiency are vulnerable to multiple infections at various anatomical sites (*17*, *23*). Overall, our report indicates that human IL-17A and IL-17F are essential for protective immunity to *C. albicans* and, to a lesser extent, *S. aureus* in the nails, skin, and oral and genital mucosae, but otherwise redundant. We

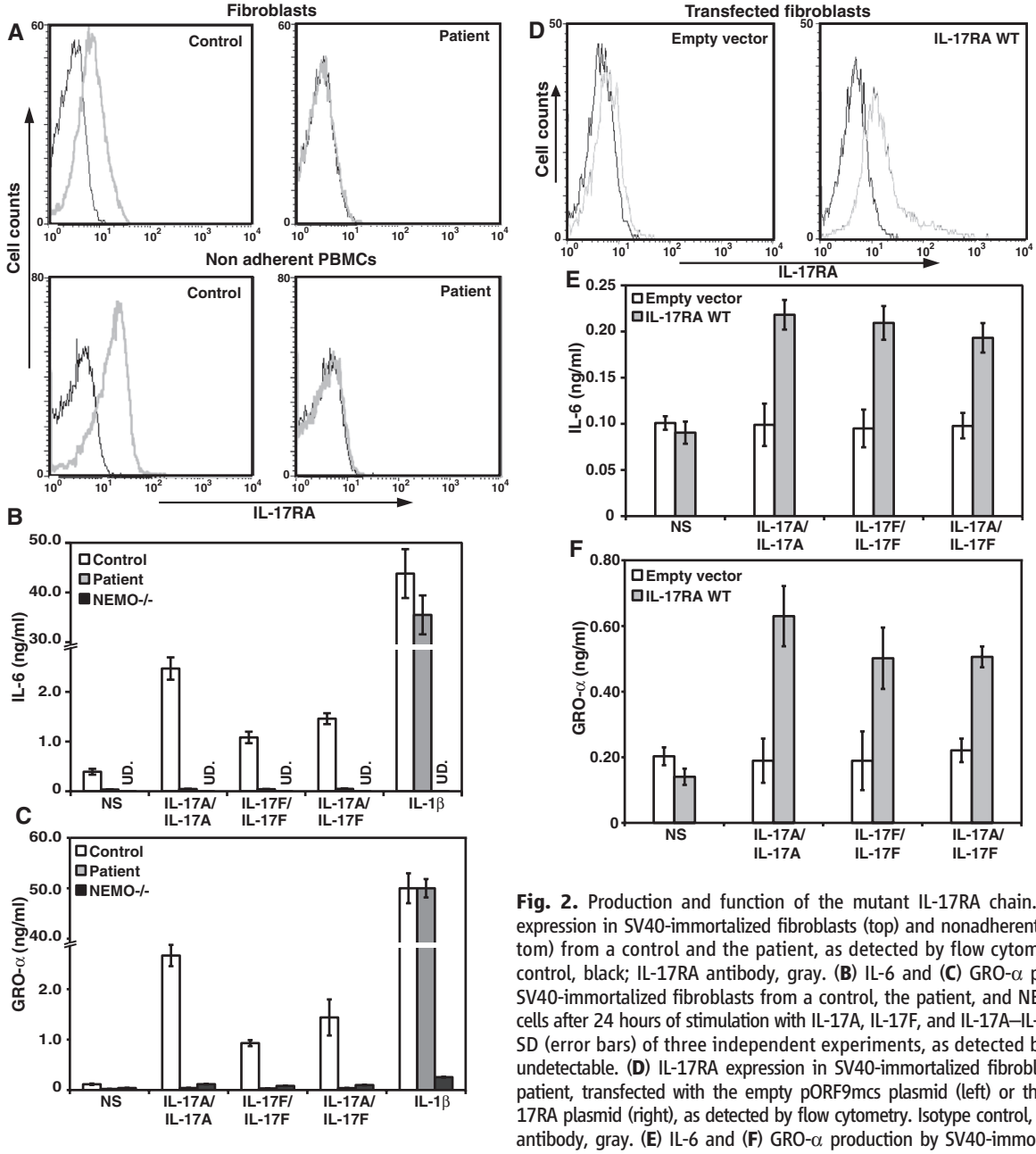

Fig. 2. Production and function of the mutant IL-17RA chain. (**A**) IL-17RA expression in SV40-immortalized fibroblasts (top) and nonadherent PBMCs (bottom) from a control and the patient, as detected by flow cytometry. Isotype control, black; IL-17RA antibody, gray. (**B**) IL-6 and (**C**) GRO-α production by SV40-immortalized fibroblasts from a control, the patient, and NEMO-deficient cells after 24 hours of stimulation with IL-17A, IL-17F, and IL-17A–IL-17F. Means ± SD (error bars) of three independent experiments, as detected by ELISA. UD, undetectable. (**D**) IL-17RA expression in SV40-immortalized fibroblasts from the patient, transfected with the empty pORF9mcs plasmid (left) or the pORF9-hIL-17RA plasmid (right), as detected by flow cytometry. Isotype control, black; IL17RA antibody, gray. (**E**) IL-6 and (**F**) GRO-α production by SV40-immortalized fibroblasts from the patient, transfected with the empty pORF9mcs plasmid (white) or the pORF9-hIL17RA plasmid (gray), after 24 hours of stimulation with IL-17A, IL-17F, and IL-17A–IL-17F. Means ± SD (error bars) of three independent experiments, as detected by ELISA.

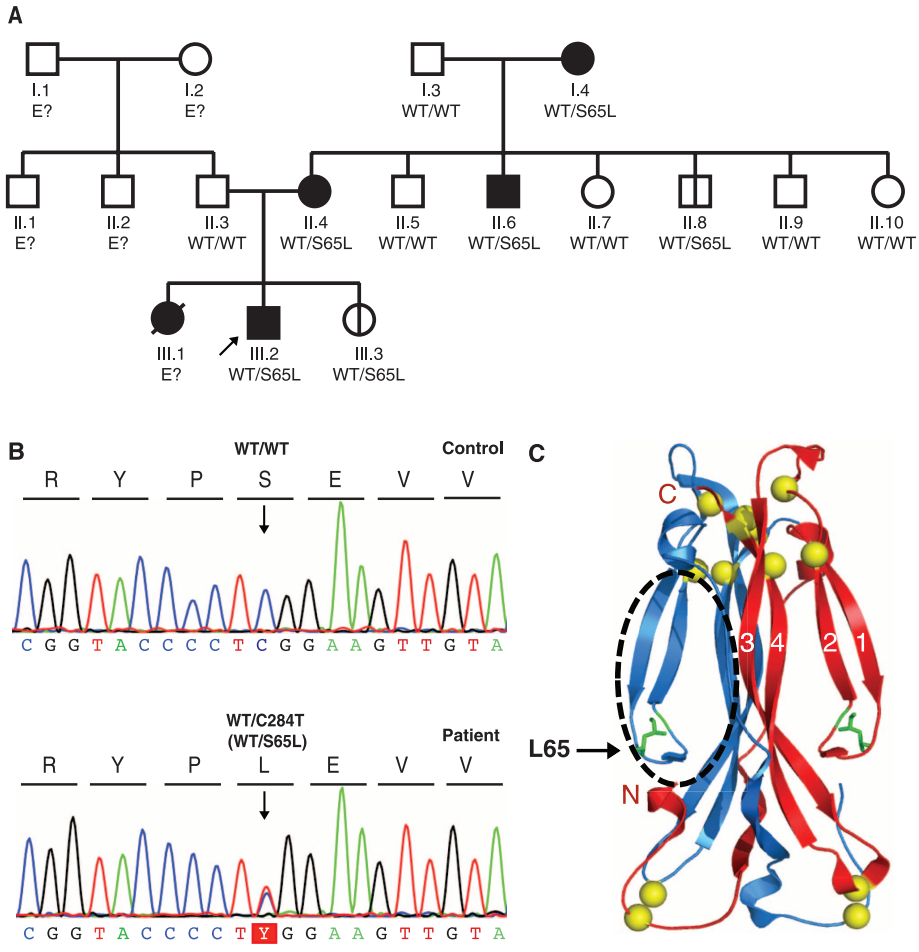

Fig. 3. A kindred with autosomal dominant IL-17F deficiency. (**A**) Family pedigree, with allele segregation. The patients, shown in black, are all heterozygous for the mutation, as is II.8, who is asymptomatic. The proband is indicated by an arrow. E? indicates individuals whose genetic status could not be evaluated. III.3 is a 9-month-old baby, also heterozygous for the mutation and currently asymptomatic. All other family members are healthy and WT for *IL17F* and are shown in white. (**B**) Heterozygous c.284C>T mutation in the patients. *IL17F* DNA sequence electrophoregrams of a control and the patient III.2. (**C**) Ribbon trace of the IL-17F dimer. Beta strands are labeled. Sulfur atoms are shown in yellow. The position of the cavity that binds to the receptor is indicated by a black circle.

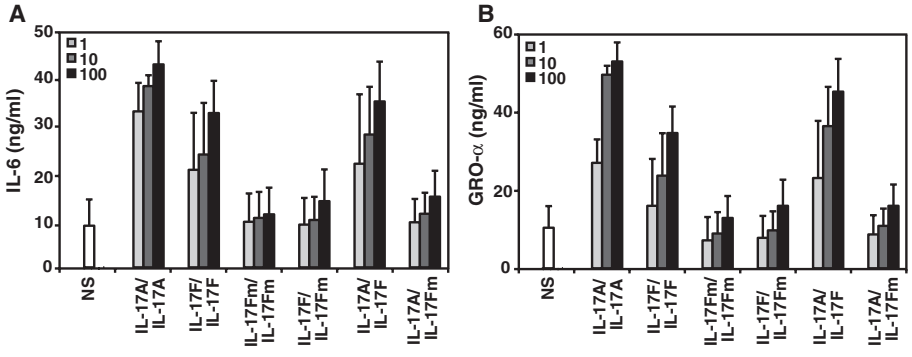

Fig. 4. Function of the mutant IL-17F protein. (**A**) Production of IL-6 and (**B**) GRO-α by control SV40 fibroblasts in response to increasing doses (ng/ml) of IL-17A, IL-17FWT, mutant IL-17F (IL-17FS65L), IL-17FWT–IL-17FS65L homodimers and of IL-17A–IL-17FWT and IL-17A–IL-17FS65L heterodimers for 24 hours. Means ± SD (error bars) of three independent experiments, as detected by ELISA.

cannot exclude the possibility that other infections may occur in patients with inborn errors of IL-17 immunity. In any event, in natura, inborn errors of IL-17 immunity clearly impair mucocutaneous immunity to *C. albicans* (*14*, *31*). Patients receiving IL-17–blocking agents should be carefully monitored, at least for mucocutaneous infections (*32*).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1200439/DC1
Materials and Methods
Figs. S1 to S13
Table S1
References

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ANNEX

Publication 2

Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis

Liu L*, Okada S*, Kong XF*, Kreins AY*, Cypowyj S*, Abhyankar A*, Toubiana J, Itan Y, Audry M, Nitschke P, Masson C, Toth B, Flatot J, Migaud M, Chrabieh M, Kochetkov T, Bolze A, Borghesi A, Toulon A, Hiller J, Eyerich S, Eyerich K, Gulácsy V, Chernyshova L, Chernyshov V, Bondarenko A, Grimaldo RM, Blancas-Galicia L, Beas IM, Roesler J, Magdorf K, Engelhard D, Thumerelle C, Burgel PR, Hoernes M, Drexel B, Seger R, Kusuma T, Jansson AF, Sawalle-Belohradsky J, Belohradsky B, Jouanguy E, Bustamante J, Bué M, Karin N, Wildbaum G, Bodemer C, Lortholary O, Fischer A, Blanche S, Al-Muhsen S, Reichenbach J, Kobayashi M, Rosales FE, Lozano CT, Kilic SS, Oleastro M, Etzioni A, Traidl-Hoffmann C, Renner ED, Abel L, Picard C, Maródi L, Boisson-Dupuis S*, Puel A*, Casanova JL*

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Gain-of-function human *STAT1* mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis

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Abbreviations used: AD, autosomal dominant; AR, autosomal recessive; CMC, chronic mucocutaneous candidiasis; CMCD, CMC disease; EMSA, electrophoretic mobility shift assay; GAS, γ-activated sequence; ISRE, IFN-stimulated response element; MSMD, Mendelian susceptibility to mycobacterial disease; WB, Western blotting.

L. Liu, S. Okada, X.-F. Kong, A.Y. Kreins, and S. Cypowyj contributed equally to this paper.
A. Abhyankar, J. Toubiana, Y. Itan, M. Audry, P. Nitschke, C. Masson, and B. Toth contributed equally to this paper.
S. Al-Muhsen, J. Reichenbach, M. Kobayashi, F. Espinoza Rosales, C. Torres Lozano, S. Sebnem Kilic, M. Oleastro, A. Etzioni, C. Traidl-Hoffmann, E.D. Renner, L. Abel, and C. Picard contributed equally to this paper.
L. Maródi, S. Boisson-Dupuis, A. Puel, and J.-L. Casanova contributed equally to this paper.

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Chronic mucocutaneous candidiasis disease (CMCD) may be caused by autosomal dominant (AD) IL-17F deficiency or autosomal recessive (AR) IL-17RA deficiency. Here, using whole-exome sequencing, we identified heterozygous germline mutations in *STAT1* in 47 patients from 20 kindreds with AD CMCD. Previously described heterozygous *STAT1* mutant alleles are loss-of-function and cause AD predisposition to mycobacterial disease caused by impaired STAT1-dependent cellular responses to IFN-γ. Other loss-of-function *STAT1* alleles cause AR predisposition to intracellular bacterial and viral diseases, caused by impaired STAT1-dependent responses to IFN-α/β, IFN-γ, IFN-λ, and IL-27. In contrast, the 12 AD CMCD-inducing *STAT1* mutant alleles described here are gain-of-function and increase STAT1-dependent cellular responses to these cytokines, and to cytokines that predominantly activate STAT3, such as IL-6 and IL-21. All of these mutations affect the coiled-coil domain and impair the nuclear dephosphorylation of activated STAT1, accounting for their gain-of-function and dominance. Stronger cellular responses to the STAT1-dependent IL-17 inhibitors IFN-α/β, IFN-γ, and IL-27, and stronger STAT1 activation in response to the STAT3-dependent IL-17 inducers IL-6 and IL-21, hinder the development of T cells producing IL-17A, IL-17F, and IL-22. Gain-of-function *STAT1* alleles therefore cause AD CMCD by impairing IL-17 immunity.

Chronic mucocutaneous candidiasis (CMC) is characterized by persistent or recurrent disease of the nails, skin, oral, or genital mucosae caused by *Candida albicans* (Puel et al., 2010b). CMC may be caused by various inborn errors of immunity. CMC is one of a multitude of infectious diseases observed in patients with broad and profound T cell deficiencies. In contrast, patients with the autosomal dominant (AD) hyper IgE syndrome, caused by dominant-negative mutations of *STAT3*, are susceptible principally to CMC and staphylococcal diseases of the lungs and skin (Minegishi, 2009). These patients have very low proportions of circulating IL-17A– and IL-22–producing T cells, probably because of impaired responses to IL-6, IL-21, and/or IL-23 (de Beaucoudrey et al., 2008; Ma et al., 2008; Milner et al., 2008; Renner et al., 2008; Minegishi et al., 2009). Patients with autosomal recessive (AR) IL-12p40 or IL-12Rβ1 deficiency suffer from Mendelian susceptibility to mycobacterial disease (MSMD) and occasionally develop mild CMC (Filipe-Santos et al., 2006; de Beaucoudrey et al., 2010). Some have low proportions of IL-17A– and IL-22–producing T cells, presumably because of the abolition of IL-23 responses (de Beaucoudrey et al., 2008, 2010). The proportion of IL-17A–producing T cells was also found to be low in a family with AR CARD9 deficiency, dermatophytosis, invasive candidiasis, and CMC (Glocker et al., 2009). Finally, CMC is the only infection in patients with autoimmune polyendocrinopathy syndrome type 1, who have high titers of neutralizing autoantibodies against IL-17A, IL-17F, and IL-22 (Kisand et al., 2010; Puel et al., 2010a). Thus, regardless of the underlying illness, CMC pathogenesis apparently involves the impairment of IL-17A, IL-17F, and IL-22 immunity (Puel et al., 2010b).

The pathogenesis of CMC was eventually deciphered through investigations of patients with CMC disease (CMCD), in which CMC is isolated, with no other infectious or autoimmune signs (Kirkpatrick, 2001; Puel et al., 2010b). The definition of CMCD is not absolute, as illustrated in some patients by cutaneous staphylococcal disease, which is milder than that in patients with AD hyper IgE syndrome (Herrod, 1990), or by autoimmune features affecting the thyroid in particular, although fewer such features are observed than in patients with autoimmune polyendocrinopathy syndrome type 1 (Atkinson et al., 2001). It is unclear whether CMCD, with these or other manifestations (Shama and Kirkpatrick, 1980; Bentur et al., 1991; Germain et al., 1994), is immunologically and genetically related to pure CMCD. Low proportions of IL-17A–producing T cells have been documented in five patients with CMCD (Eyerich et al., 2008). Moreover, a candidate gene approach centered on IL-17 immunity recently revealed the first genetic etiologies of pure CMCD. In a consanguineous family from Morocco, a child with CMCD was found to display AR complete IL-17RA deficiency (Puel et al., 2011). His leukocytes and fibroblasts did not respond to IL-17A or IL-17F homodimers, or to IL-17A/F heterodimers. Four patients from an Argentinean family were shown to harbor dominant-negative mutations in the *IL17F* gene (Puel et al., 2011). Mutated IL-17F–containing homodimers and heterodimers were produced in normal amounts but were not biologically active, as they were unable to bind to the IL-17 receptor. Morbid mutations in *IL17RA* and *IL17F* demonstrated that CMCD could be caused by inborn errors of IL-17 immunity. However, no genetic etiology has yet been identified for most patients with CMCD. We set out to identify new genetic etiologies of CMCD through a recently developed genome-wide approach based on whole-exome sequencing (Alcaïs et al., 2010; Bolze et al., 2010; Byun et al., 2010; Ng et al., 2010).

RESULTS

We investigated one sporadic case and the probands from five multiplex kindreds with AD CMCD, by whole-exome sequencing. The annotated data were analyzed with sequence analysis software that had been developed in-house and made it possible to analyze and compare several exome sequences simultaneously. A hierarchy of candidate variations was generated by filtering out known polymorphisms reported in dbSNP and 1,000-genome databases. We also used our own database of 250 exomes to filter out unreported polymorphisms (Table S1). The only relevant gene displaying heterozygous variations in at least four of the six unrelated patients with AD CMCD was *STAT1* (Fig. 1, A and B, Kindreds A, B, G, and L; Table I; and Table S2). Three different *STAT1* mutations were found in four patients; they were confirmed by Sanger

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sequencing and shown to be missense mutations. All these mutations affected the coiled-coil domain, which plays a key role in unphosphorylated STAT1 dimerization and STAT1 nuclear dephosphorylation (Fig. 1, A and C; Chen et al., 1998; Levy and Darnell, 2002; Braunstein et al., 2003; Zhong et al., 2005; Hoshino et al., 2006; Mertens et al., 2006). We therefore sequenced the corresponding coding region of *STAT1* (exons 6 to 10) in another 106 patients, including 57 with sporadic CMCD and 49 from 22 multiplex kindreds with AD CMCD. 29 patients from 16 kindreds were heterozygous for a *STAT1* missense mutation (Fig. 1, A and B, Kindreds C-F, H-K, and M-T; Fig. 1 C; and Table I; Table S3). In total, 36 patients from 20 kindreds were heterozygous for 1 of the 12 missense mutations identified that affected the coiled-coil domain of STAT1. 11 other CMCD patients in these kindreds were not genotyped. The intrafamilial segregation of the mutations was consistent with an AD trait, as all patients with CMCD from the kindreds tested were heterozygous, whereas none of these mutations was found in the heterozygous state in any of the healthy relatives tested (Fig. 1 B). Moreover, the *STAT1* haplotypes for common SNPs indicated that the five recurrent mutations were caused by mutation hotspots rather than founder effects (unpublished data). Finally, the mutations were found to have occurred de novo in at least four kindreds, which is consistent with a high clinical penetrance of these alleles. The mutations were not found in the National Center for Biotechnology Information, Ensembl, and dbSNP databases. They were also absent from 1,052 controls from 52 ethnic groups in the Centre d'Etude du Polymorphisme Humain and Human Genome Diversity panels, suggesting that they were rare, CMCD-inducing variants rather than irrelevant polymorphisms.

The 12 missense mutations were not conservative and were therefore predicted to affect protein structure and function. Moreover, most of the affected residues were found to have been conserved throughout evolution in the species in which *STAT1* had been sequenced (Table S3). Accordingly, POLYphen II predicted that all but one of these mutations would be possibly or probably damaging (Adzhubei et al., 2010; Table S3). None of the previously described nine patients with AD STAT1 deficiency and MSMD was heterozygous for mutations affecting the coiled-coil domain (Fig. 1, A and C; Dupuis et al., 2001; Chapgier et al., 2006a; Averbuch et al., 2011; unpublished data). However, three of the eight patients with AR STAT1 deficiency and susceptibility to intracellular bacterial and viral diseases, who, like their heterozygous relatives, did not display CMC, carried mutations affecting the coiled-coil domain (Fig. 1, A and C; Chapgier et al., 2009; Chapgier et al., 2006b; Dupuis et al., 2003; Kong et al., 2010; Kristensen et al., 2011; Averbuch et al., 2011). These three patients from two kindreds carried the K201N or K211R mutation (Kong et al., 2010; Kristensen et al., 2011). Nevertheless, the three-dimensional structure of phosphorylated STAT1 molecules revealed that the 12 CMCD-linked missense mutations affected a cluster of residues located in a specific pocket of the coiled-coil domain, near residues essential for STAT1 dephosphorylation (Fig. 1 C; Chen et al., 1998; Zhong et al., 2005; Mertens et al., 2006). In contrast, the other two morbid mutations (K201N and K211R) affect residues located on the other side of the coiled-coil domain (Fig. 1 C). Moreover, these two hypomorphic alleles were shown to be pathogenic not because they were missense, but because they promoted the splicing out of exon 8, resulting in AR partial STAT1 deficiency, with the production of small amounts of intrinsically functional STAT1 molecules (Kong et al., 2010; Kristensen et al., 2011). These genetic data strongly suggest that heterozygous missense mutations in the coiled-coil domain of STAT1 may cause AD CMCD in a large fraction of patients. Nevertheless, the occurrence of other germline mutations in *STAT1* in patients without CMC and with an AD or AR predisposition to other infectious diseases raised questions about whether these mutations were really responsible for CMCD and the underlying mechanism of disease.

We functionally characterized the CMCD-causing *STAT1* allele R274Q, which was found in four kindreds (Fig. 1 B and Table I). We compared it with a WT and an MSMD-causing loss-of-function *STAT1* allele (L706S; Dupuis et al., 2001). We transfected STAT1-deficient U3C fibrosarcoma cells with WT, R274Q, or L706S *STAT1* alleles. Upon stimulation with IFN-α, IFN-γ, or IL-27, cells transfected with the R274Q allele responded two to three times more strongly than those transfected with the WT allele, as shown by measurement of the induction of γ-activated sequence (GAS)–dependent reporter gene transcription activity, with mock- and L706S-transfected cells serving as negative controls (Fig. 2 A and Fig. S1 A). All *STAT1* alleles were expressed at an equal strength, as shown by Western blotting (WB; Fig. 2 B). Higher levels of STAT1 phosphorylation were observed for the R274Q allele than for the WT allele after stimulation with IFN-γ, IFN-α, and IL-27, whereas STAT3 phosphorylation levels were similar for the two alleles (Fig. 2 B). In contrast, the induction of IFN-stimulated response element (ISRE)–dependent transcription activity by IFN-α was normal (Fig. S1, B and C). In the same experimental conditions, the other 10 CMCD-associated *STAT1* alleles tested were also gain-of-function, unlike the K201N and K211R alleles (Fig. S1 D). Upon stimulation with IFN-γ, IFN-α, or IL-27, an increase in GAS-binding activity was detected in cells transfected with the R274Q allele (Fig. S1 E). Accordingly, the transcription of the *CXCL9* and *CXCL10* target genes was enhanced (Fig. 2, C and D). Overall, these data indicate that at least 11 of the 12 CMCD-linked *STAT1* missense alleles are intrinsically gain-of-function.

The mechanism involved an increase in STAT1 tyrosine 701 residue phosphorylation, as shown for R274Q by WB after stimulation with IFN-α, IFN-γ, and IL-27 (Fig. 2 B). STAT1 was not constitutively activated, and STAT3 was normally activated in R274Q-transfected cells (Fig. 2 B and not depicted). Almost all the mutant STAT1 molecules, which were phosphorylated in response to IFN-γ, translocated to and accumulated in the nucleus, as shown by immunofluorescence (Fig. S1 F). WB showed R274Q STAT1 to be more

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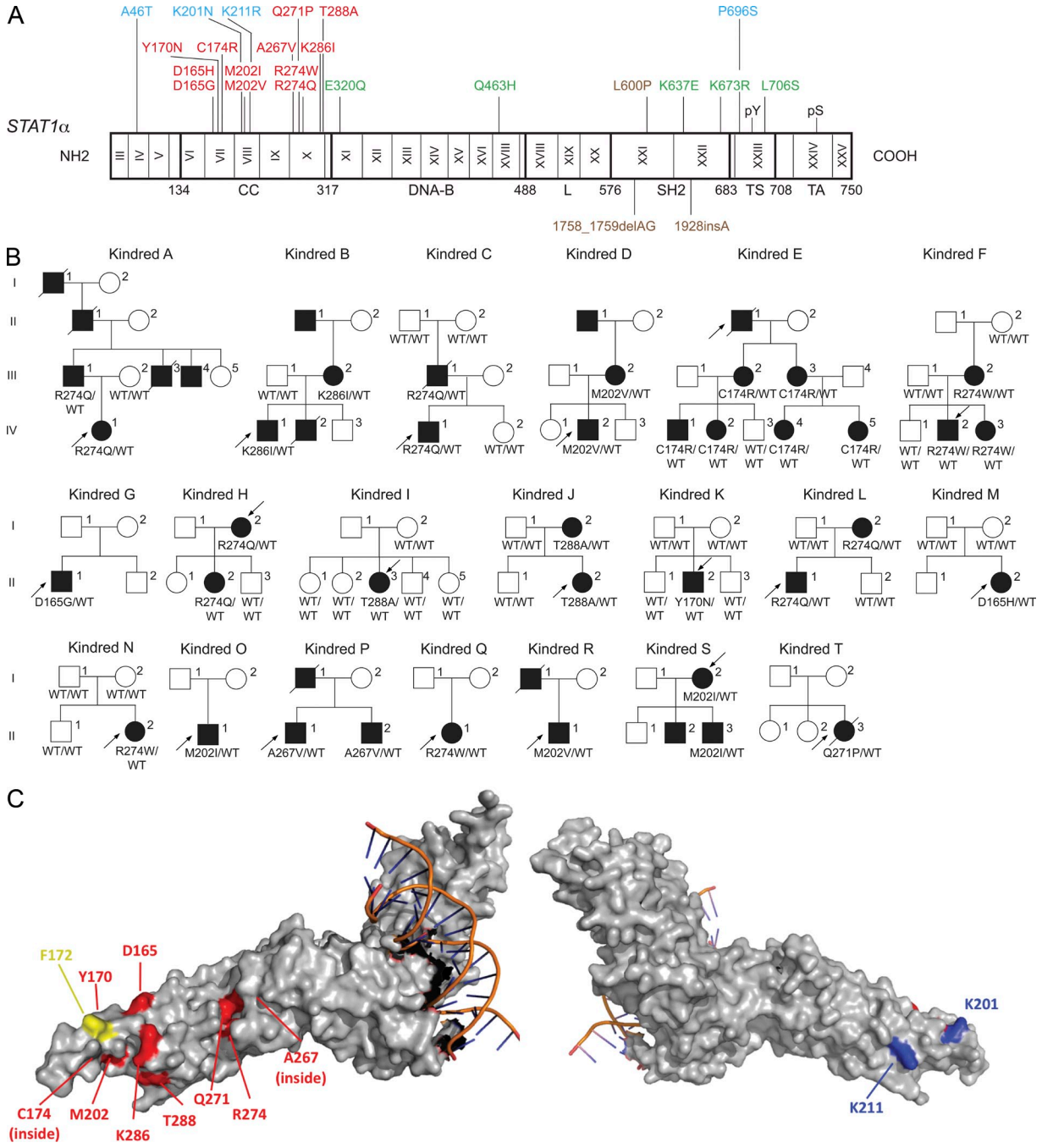

Figure 1. Heterozygous missense mutations affecting the STAT1 coiled-coil domain in kindreds with AD CMCD. (A) The human *STAT1* α isoform is shown, with its known pathogenic mutations. Coding exons are numbered with roman numerals and delimited by a vertical bar. Regions corresponding to the coiled-coil domain (CC), DNA-binding domain (DNA-B), linker domain (L), SH2 domain (SH2), tail segment domain (TS), and transactivator domain (TA) are indicated, together with their amino-acid boundaries, and are delimited by bold lines. Tyr701 (pY) and Ser727 (pS) are indicated. Mutations in green are dominant and associated with partial STAT1 deficiency and MSMD. Mutations in brown are recessive and associated with complete STAT1 deficiency and intracellular bacterial and viral disease. Mutations in blue are recessive and associated with partial STAT1 deficiency and intracellular bacterial and/or viral disease. Mutations in red are dominant and associated with a gain-of-function of STAT1 and CMCD. (B) Pedigrees of 20 families with AD "gain-of-function" *STAT1* mutations. Each kindred is designated by a letter (A to T), each generation is designated by a roman numeral (I-II-III-IV), and each individual is designated by an Arabic numeral (each individual studied is identified by a code of this type, organized from left to right). Black indicates CMCD patients. The probands are indicated by arrows. When tested, the genotype for *STAT1* is indicated below each individual. (C) Three-dimensional structure of phosphorylated STAT1 in complex with DNA. Connolly surface representation, with the following amino acids highlighted: red, amino acids mutated in patients with CMCD; blue, amino acids located in the coiled-coil domain and mutated in patients with MSMD and viral diseases; yellow, amino acids identified in vitro as affecting the dephosphorylation process.

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Table I. Summary of the clinical and genetic data for the patients

Patient	Age at presentation	Origin	Clinical features of CMC	Cause of death (age/yr)	Autoimmunity	Genotype
A-I-1	-	France	Nails	Not related to the disease (old age)	None	-
A-II-1	-	France	Nails	Not related to the disease (old age)	None	-
A-III-1	1 mo	France	Nails, oral cavity, oropharynx, genital mucosa		None	WT/R274Q
A-III-3	-	France	Nails, oral cavity	Not related to the disease (40)	None	-
A-III-4	-	France	Nails, oral cavity		None	-
A-IV-1	1 mo	France	Nails, oral cavity, oropharynx		None	WT/R274Q
B-II-1	-	France	-		None	-
B-III-2	3 yr	France	Skin, nails, oral cavity, oropharynx, genital mucosa		None	WT/K286I
B-IV-1	5 yr	France & Congo	Skin, nails, oral cavity, oropharynx		None	WT/K286I
B-IV-2	5 mo	France & Congo	Skin, nails, oral cavity, oropharynx	Cerebral aneurysm (8)	None	-
C-III-1	-	Turkey	Nails, oral cavity, genital mucosa	Cerebral aneurysm (34)	Thyroid autoimmunity	WT/R274Q
C-IV-1	-	Turkey	Nails, oral cavity		None	WT/R274Q
D-II-1	-	France	Nails, oral cavity, genital mucosa		-	-
D-III-2	7 yr	France	Skin, oral cavity, oropharynx		None	WT/M202V
D-IV-2	1 mo	France	Skin, nails, oropharynx		Thyroid autoimmunity	WT/M202V
E-II-1	1 yr	Germany	Skin, oral cavity, oropharynx	Squamous cell carcinoma (54)	-	-
E-III-2	1 yr	Germany	Nails, oral cavity, oropharynx, genital mucosa		Thyroid autoimmunity	WT/C174R
E-III-3	9 mo	Germany	Skin, nails, oral cavity, oropharynx, genital mucosa		Thyroid autoimmunity	WT/C174R
E-IV-1	18 mo	Germany	Skin, oral cavity, oropharynx, genital mucosa		None	WT/C174R
E-IV-2	2 yr	Germany	Skin, oral cavity, oropharynx		Thyroid autoimmunity	WT/C174R
E-IV-4	2 yr	Germany	Skin, oral cavity, oropharynx, genital mucosa		None	WT/C174R
E-IV-5	1 yr	Germany	Skin, nails, oral cavity, oropharynx		None	WT/C174R
F-III-2	1 mo	Argentina	Nails, oral cavity, oropharynx, genital mucosa		-	WT/R274W
F-IV-2	1 mo	Argentina	Skin, nails, oral cavity, oropharynx		-	WT/R274W
F-IV-3	6 mo	Argentina	Nails, oral cavity, genital mucosa		-	WT/R274W
G-II-1	3 mo	Ukrainian	Nails, skin, oral cavity, oropharynx, esophagus		None	WT/D165G
H-I-2	1 yr	Japan	Skin, oropharynx, esophagus		-	WT/R274Q
H-II-2	5 yr	Japan	Oral cavity, oropharynx		-	WT/R274Q
I-II-3	9 mo	Mexico	Skin, nails, oral cavity, genital mucosa		None	WT/T288A
J-I-2	-	Switzerland	Oral cavity, oropharynx		None	WT/T288A
J-II-2	3 mo	Switzerland	Oral cavity, oropharynx		-	WT/T288A
K-II-2	11 mo	Switzerland	Nails, oral cavity, oropharynx		Thyroid autoimmunity	WT/Y170N
L-I-2	7 yr	France	Skin, nails, oropharynx, esophagus		Thyroid autoimmunity	WT/R274Q
L-II-1	1 mo	France	Skin, nails, oropharynx, esophagus		None	WT/R274Q
M-II-2	6 mo	Germany	Skin, nails, oropharynx, genital mucosa		Thyroid autoimmunity	WT/D165H

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Table I. Summary of the clinical and genetic data for the patients *(Continued)*

Patient	Age at presentation	Origin	Clinical features of CMC	Cause of death (age/yr)	Autoimmunity	Genotype
N-II-2	1 yr	Germany	Skin, nails, oropharynx	Squamous cell carcinoma (54)	None	WT/R274W
O-II-1	18 mo	Germany	Oral cavity, oropharynx		None	WT/M202I
P-I-1	1 yr	Israel	Oropharynx, genital mucosa	Not related to the disease (46)	None	-
P-II-1	<2 yr	Israel	Skin, nails, oropharynx		None	WT/A267V
P-II-2	<2 yr	Israel	Skin, nails, oropharynx		None	WT/A267V
Q-II-1	1 mo	France	Skin, oral cavity, oropharynx, genital mucosa		None	WT/R274W
R-I-1	4 yr	France	Skin, nails, oropharynx	Squamous cell carcinoma (55)	None	-
R-II-1	18 mo	France	Lips, oropharynx		None	WT/M202V
S-I-2	6 mo	France	Skin, oral cavity, oropharynx		Systemic lupus erythematosus	WT/M202I
S-II-2	1 yr	France	Nails		None	-
S-II-3	1 mo	France	Skin, oropharynx		None	WT/M202I
T-II-3	1 yr	Germany	Skin, nails, oropharynx	Squamous cell carcinoma (41)	None	WT/Q271P

None of the patients displays autoantibodies against IL-17A, IL-17F, and IL-22. -, unknown.

strongly phosphorylated than the WT protein in both cytoplasmic and nuclear extracts (Fig. S1 G). The mechanism underlying the gain of R274Q phosphorylation was explored with the tyrosine kinase inhibitor staurosporine and the phosphatase inhibitor pervanadate. The dephosphorylation of IFN-γ–activated R274Q STAT1 was impaired by staurosporine, but less than that of the known dephosphorylation mutant F77A (Fig. 2 E; Zhong et al., 2005). In contrast, pervanadate normalized the phosphorylation of R274Q to WT levels (Fig. 2 F). Another CMCD-linked mutation, D165G (Fig. 1, A–C), also resulted in impaired dephosphorylation that could be normalized by adding pervanadate (Fig. 2 F and Fig. S1 H). Thus, at least two CMCD-linked *STAT1* missense alleles (R274Q and D165G) are gain-of-function caused by the impairment of nuclear dephosphorylation. These alleles may therefore enhance cellular responses to cytokines activating STAT1 predominantly and STAT3 to a lesser extent, such as IFN-α/β, IFN-γ, IFN-λ, and IL-27, and possibly also responses to cytokines activating STAT3 predominantly and STAT1 to a lesser extent, such as IL-6, IL-21, IL-22, and IL-23 (Fig. S2).

We investigated the dominance of the *STAT1* alleles at the cellular level by testing EBV-B–transformed (EBV-B) cells and SV-40–transformed dermal fibroblasts from a CMCD patient heterozygous for the *STAT1* R274Q allele. We observed enhanced IFN-α/β–, IFN-γ–, and IL-27–dependent STAT1 phosphorylation in EBV-B cells from a patient heterozygous for the *STAT1* R274Q allele, as shown by WB (Fig. 3, B and D). Phospho-STAT1 accumulated in the nucleus of R274Q heterozygous SV-40 fibroblasts upon IFN-γ stimulation, as well as in EBV-B cells (Fig. 3 I and Fig. S3 D). Moreover, the IFN-α/β–, IFN-γ–, and IL-27–induced DNA-binding activity of GAF was stronger in cells from the CMCD patient than in those from a healthy control or from a MSMD patient carrying the L706S mutant allele, as shown by electrophoretic mobility shift assay (EMSA; Fig. 3, A and C). In contrast, the DNA-binding activity of ISGF-3 seemed to be normal in cells from the patient stimulated with IFN-α/β (Fig. S3 A). These data strongly suggest that the heterozygous R274Q allele is dominant for STAT1-dependent responses and gain-of-function for GAF-dependent cellular responses to key STAT1-activating cytokines, such as IFN-α/β, IFN-γ, and IL-27. The mutation may also affect IFN-λ responses.

We then tested cytokines that predominantly activate STAT3, rather than STAT1, such as IL-6, IL-21, IL-22, and IL-23 (Hunter, 2005; Kishimoto, 2005; Kastelein et al., 2007; Spolski and Leonard, 2008; Donnelly et al., 2010; Sabat, 2010; Ouyang et al., 2011). Peripheral T cell blasts from a patient displayed normal STAT3 activation in response to IL-23, as shown by WB (Fig. S3 B). No increase in STAT1 phosphorylation was detected in cells from a patient or controls upon IL-23 stimulation. Furthermore, fibroblasts from a patient displayed normal activation of STAT3 in response to IL-22 (Fig. S3 C). In the same conditions, no STAT1 phosphorylation was detected in cells from the patient or controls (unpublished data). In contrast, the levels of STAT1 phosphorylation in response to IL-6 and IL-21 were higher in the patient's EBV-B cells than in cells from healthy controls and from a patient with MSMD heterozygous for the L706S allele, whereas STAT3 activation was normal as shown by WB (Fig. 3, F and H). Consistent with these findings, stronger GAS activity was observed in cells from the patient in response to IL-6 and IL-21 stimulation (Fig. 3, E and G). These data suggest that heterozygous missense mutations in the coiled-coil domain of STAT1 are dominant and gain-of-function for GAF-dependent cellular responses for cytokines that predominantly activate STAT3, such as IL-6 and IL-21. Overall, these data suggest that the *STAT1* alleles are truly responsible for CMCD in these kindreds and raise questions about the immunological basis of CMCD.

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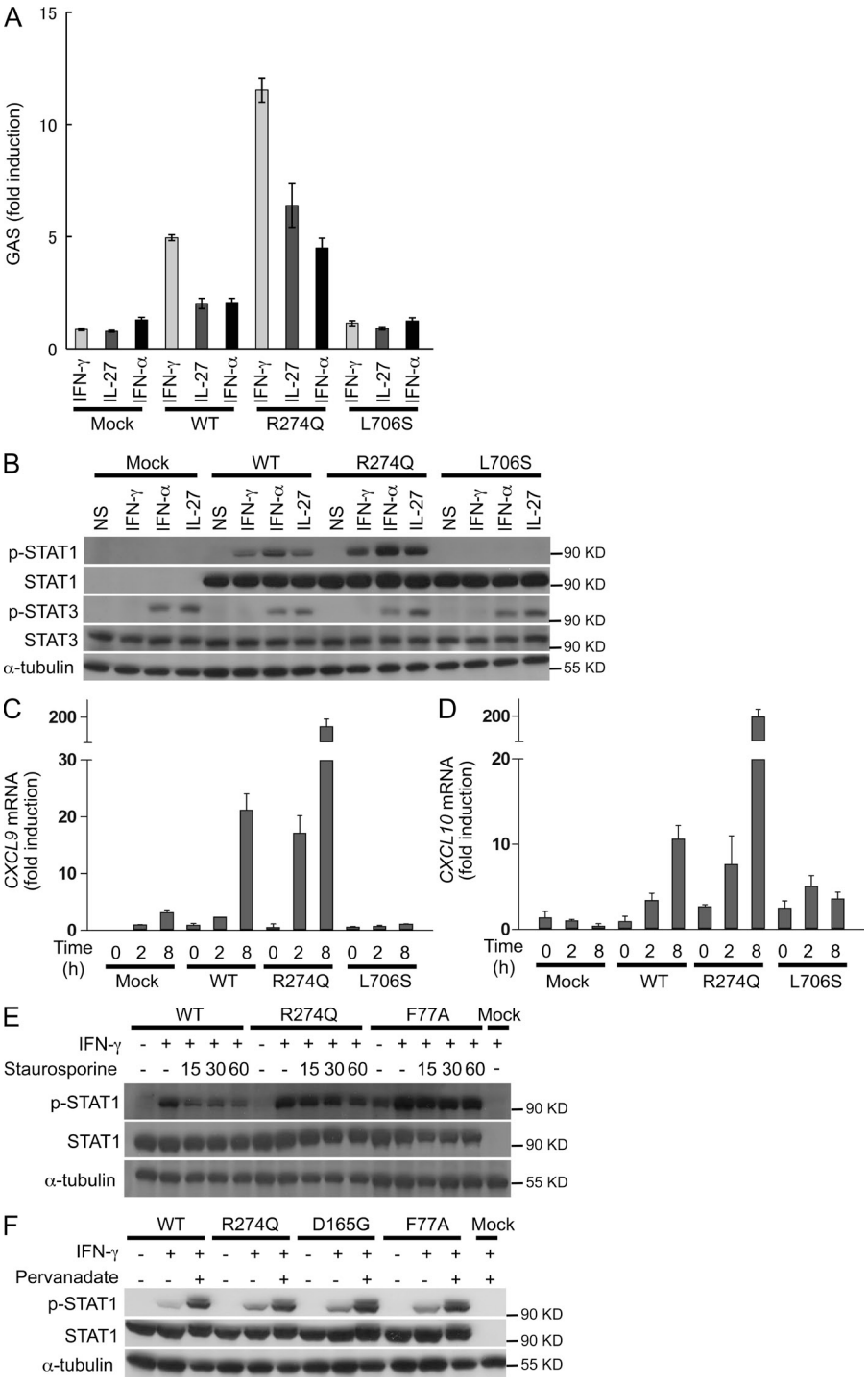

Figure 2. The mutant R274Q *STAT1* allele is gain-of-phosphorylation and gain-of-function for GAF-dependent cellular responses. U3C cells were transfected with a mock vector, a WT, or two mutant alleles of *STAT1* (R274Q and L706S). The response to IFN-γ, IL-27, and IFN-α was then evaluated by determining luciferase activity of a reporter gene under the control of the GAS promoter (A), and by determining STAT1 and STAT3 phosphorylation by Western blot (B). Experiments were performed at least three times independently. (C and D) Quantitative RT-PCR was used to measure the induction of *CXCL9* (C) and *CXCL10* (D) 2–8 h after stimulation with IFN-γ. Experiments were performed two times independently. (E) The nuclear dephosphorylation of STAT1 was tested by WB in U3C cells transfected with a mock vector, WT *STAT1*, the R274Q, or the F77A (a known loss-of-dephosphorylation mutant) *STAT1* mutant alleles, and treated with IFN-γ with or without the tyrosine kinase inhibitor staurosporine for the indicated periods of time (in minutes). Three independent experiments were performed. (F) Western blot of U3C cells transfected with mock, WT, R274Q, D165G, and F77A alleles of *STAT1*, nontreated or treated with IFN-γ in the absence or presence of the phosphatase inhibitor pervanadate. Two independent experiments were performed. Error bars represent SD of one experiment done in triplicate (Fig. S1 D).

IL-27 is a potent inhibitor of the development of IL-17–producing T cells in mice (Batten et al., 2006; Stumhofer et al., 2006; Yoshimura et al., 2006; Amadi-Obi et al., 2007; Diveu et al., 2009; El-behi et al., 2009; Villarino et al., 2010) and humans (Diveu et al., 2009; Liu and Rohowsky-Kochan, 2011), through a mechanism dependent on STAT1 (Amadi-Obi et al., 2007; Batten et al., 2006; Diveu et al., 2009; Liu and Rohowsky-Kochan, 2011; Stumhofer et al., 2006; Villarino et al., 2010). Moreover, mouse IFN-γ (Feng et al., 2008; Tanaka et al., 2008; Villarino et al., 2010) and human IFN-α/β (Chen et al., 2009; Ramgolam et al., 2009) have been shown to antagonize the development of IL-17–producing T cells via STAT1. In addition, IL-6, IL-21, and IL-23 are prominent inducers of IL-17–producing T cells, via a mechanism dependent on STAT3 and antagonized by STAT1 (Hirahara et al., 2010). Finally, we recently showed that inborn errors of IL-17F or IL-17RA were genetic etiologies of CMCD (Puel et al., 2010b, 2011). We thus determined the proportion of IL-17A– and IL-22–producing T cells by flow cytometry in patients with heterozygous *STAT1* mutations and AD CMCD. The 18 CMCD patients carrying gain-of-function mutations in *STAT1* that were tested had lower proportions of circulating IL-17A– and IL-22–producing T cells ex vivo than 28 healthy controls ($P < 10^{-4}$) and six patients bearing loss-of-function *STAT1* alleles ($P < 2.10^{-3}$; Fig. 4, A and B; and Fig. S4 G). In contrast, they displayed normal proportions of IFN-γ–producing T cells (Fig. S4 F).

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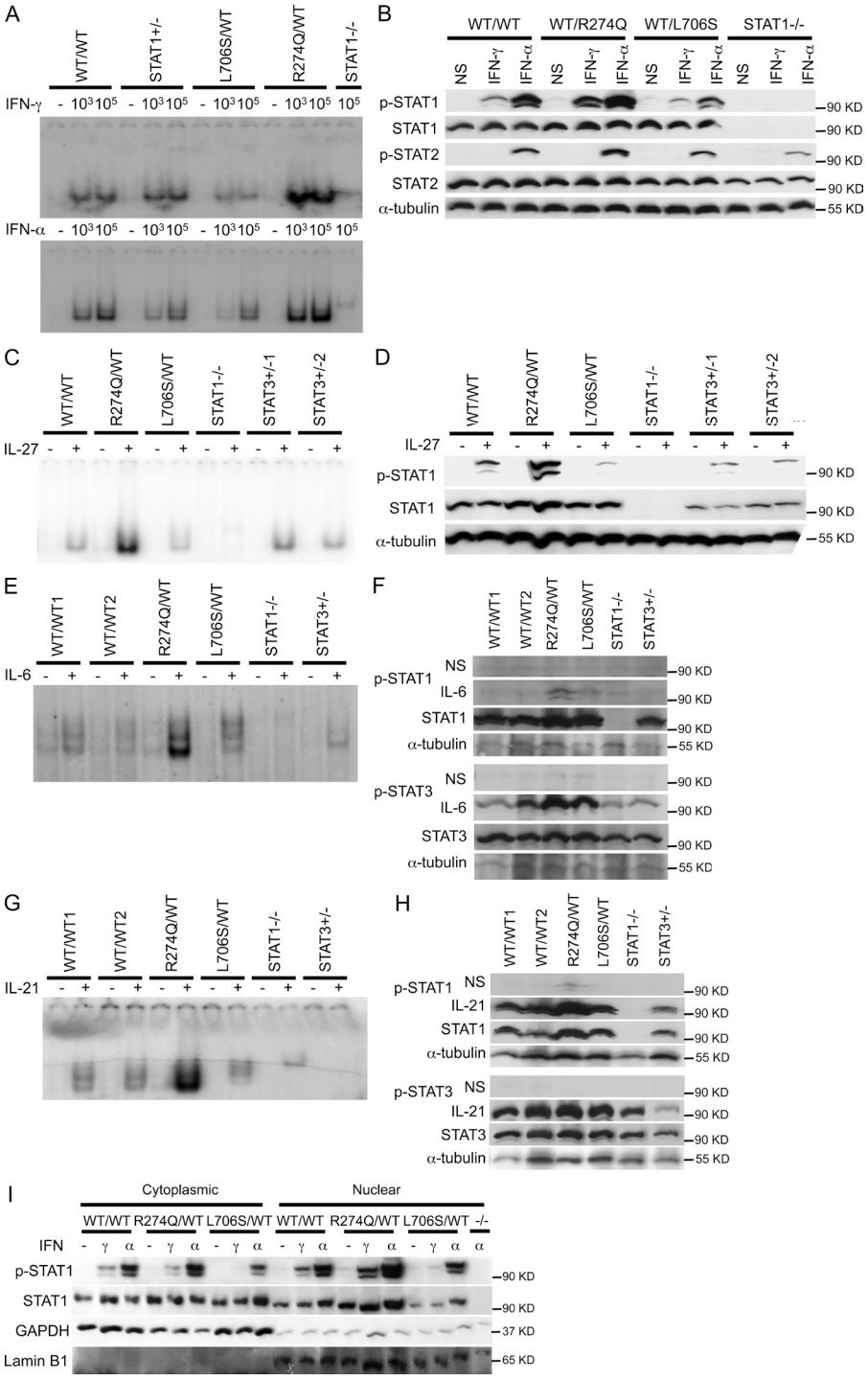

Figure 3. The mutant R274Q *STAT1* allele is dominant for GAF-dependent cellular responses at the cellular level. The responses of the patient's EBV-B cells (R274Q/WT) were evaluated independently at least twice, by EMSA, with a GAS probe (A, C, E, and G), and by Western blot (B, D, F, and H). This response was compared with that of one or two healthy controls (WT/WT1 and WT/WT2), heterozygous cells with a WT and a loss-of-function *STAT1* allele (STAT1$^{+/-}$), cells heterozygous for a dominant loss-of-function mutation of *STAT1* (L706S/WT), cells with complete STAT1 deficiency (STAT1$^{-/-}$), and cells from two patients heterozygous for dominant loss-of-function mutations of *STAT3* (STAT3$^{+/-1}$ and STAT3$^{+/-2}$). Cells were left nonstimulated (NS) or stimulated, as indicated, with IFN-γ, IFN-α, IL-27, IL-6, and IL-21. pSTAT is an antibody specific for STAT with a phosphorylated tyrosine residue. (I) The nuclear and cytoplasmic fractions of EBV-B cells from a control (WT/WT), a CMCD patient (R274Q/WT), a heterozygous patient with a dominant loss-of-function mutation of *STAT1* (L706S/WT) and a patient with complete STAT1 deficiency ($^{-/-}$) stimulated with IFN-γ and IFN-α were tested for the presence of phosphorylated STAT1 and STAT1 by WB. Antibodies directed against GAPDH and Lamin B1 were used to normalize the amount of cytoplasmic and nuclear proteins, respectively. The experiment was performed twice.

Moreover, only very small amounts of IL-17A, IL-17F, and IL-22 were secreted by freshly prepared leukocytes after ex vivo stimulation with PMA and ionomycin ($P < 8.10^{-3}$), as shown by ELISA (Fig. 4, C–E). In contrast, the amounts of secreted IL-17A, IL-17F, and IL-22 were normal in patients heterozygous or homozygous for loss-of-function or hypomorphic *STAT1* mutations (Fig. 4, C–E). Interestingly, in all assays, the proportions of IL-17A– and IL-22–producing T cells and the amounts of IL-17A, IL-17F, and IL-22 secreted were smallest for the four patients with the most apparently severe clinical phenotype (Fig. 4, A–E and not depicted).

After the culture of PBMCs in vitro in the presence of various cytokines, including IL-6, TGF-β, IL-1β, and IL-23, the proportion of IL-17A– and IL-22–producing T cell blasts remained significantly lower ($P < 10^{-4}$) in CMCD patients carrying *STAT1* mutations than in controls (Fig. S4, A and B; and not depicted). In contrast, the proportions of IL-17A– and IL-22–producing T cell blasts were normal in patients with loss-of-function *STAT1* mutations (Fig. S4, A and B; and not depicted). The amounts of IL-17A, IL-17F, and IL-22 in the supernatant of T cell blasts stimulated with PMA and ionomycin after culture in vitro were also significantly lower in patients with *STAT1* mutations and CMCD ($P < 4.10^{-4}$; Fig. S4, C–E; and not depicted). In contrast, patients with loss-of-function mutant *STAT1* alleles displayed normal levels of cytokine secretion (Fig. S4, C–E; and not depicted). Finally, levels of IL-12p70 and

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IL-12p40 production by whole blood stimulated with IFN-γ were higher in CMCD patients bearing gain-of-function *STAT1* alleles than in patients bearing loss-of-function *STAT1* alleles and healthy controls (Fig. 4 F and not depicted). Thus, patients with familial or sporadic AD CMCD heterozygous for mutations affecting the coiled-coil domain of STAT1, including the dominant gain-of-function R274Q mutant allele, displayed lower levels of IL-17 cytokine production by peripheral T cells, providing a molecular mechanism for the disease.

DISCUSSION

We have shown that several germline missense mutations affecting the coiled-coil domain of STAT1 may cause sporadic and familial AD CMCD. The underlying mechanism involves a gain of STAT1 phosphorylation caused by the loss of nuclear dephosphorylation, resulting in a gain-of-function of GAF in response to various cytokines. Impaired dephosphorylation may not be the only mechanism influencing the impact of these mutations on the transcription of STAT1 target genes, as these mutations may also affect other processes, such as the dimerization of unphosphorylated STAT1. Moreover, the gain-of-function, which manifests itself in terms of DNA-binding activity, reporter gene induction, and target gene induction, may not necessarily increase the transcription of all target genes, possibly even resulting in the repression of some genes. In addition, the various *STAT1* mutations, although they all affect the coiled-coil domain and are probably all loss-of-dephosphorylation and gain-of-function, may somewhat differ from each other in terms of their functional impact. The genome-wide impact of these mutations on the transcriptome remains to be assessed in various cell types stimulated with a range of cytokines. In any case, the gain-of-function mutant *STAT1* alleles were dominant for GAF activation in all cell types tested. They affected cellular responses to various cytokines, including IFN-α/β, IFN-γ, and IL-27, which predominantly activate STAT1 over STAT3, and IL-6 and IL-21, which predominantly activate STAT3 over STAT1. These mutations probably also strengthen cellular responses to IFN-λ. However, they do not seem to affect STAT1-containing ISGF-3 activation by IFN-α/β, at least in the conditions tested. Moreover, STAT3 activation by IL-6, IL-21, IL-22, and IL-23 is maintained, suggesting that STAT3 activation by IL-26 is also intact.

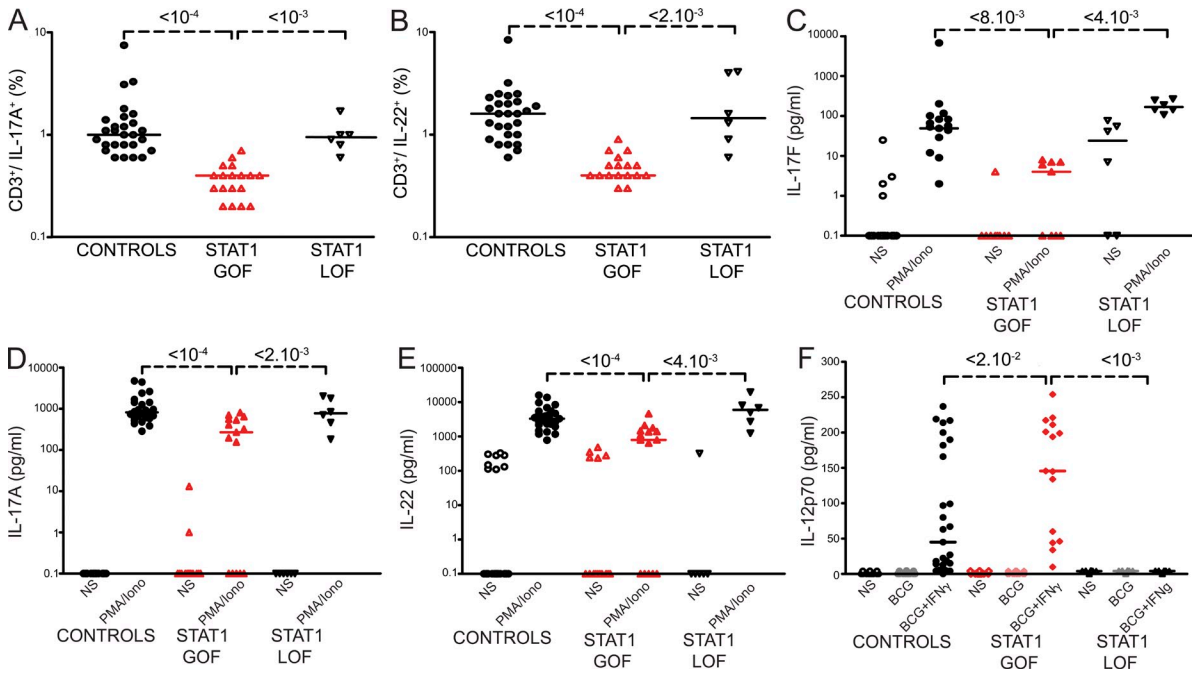

Figure 4. Impaired development and function of IL-17– and IL-22–producing T cells ex vivo in patients with AD CMCD and *STAT1* mutations. Each symbol represents a value from a healthy control individual (black circles), a patient bearing a *STAT1* gain-of-function (GOF) allele (red upright triangles), or a patient bearing one or two *STAT1* loss-of-function (LOF) alleles (black upside-down triangles). (A and B) Percentage of $CD3^+/IL\text{-}17A^+$ (A) and $CD3^+/IL\text{-}22^+$ (B) cells, as determined by flow cytometry, in nonadherent PBMCs activated by incubation for 12 h with PMA and ionomycin. (C–E) Secretion of IL-17F (C), IL-17A (D) and IL-22 (E) by whole blood cells, as determined by ELISA, in the absence of stimulation (open symbols) and after stimulation with PMA and ionomycin for 48 h (closed symbols). Horizontal bars represent medians. The p-values for the nonparametric Wilcoxon test, between patients with *STAT1* GOF mutations ($n = 18$) and controls ($n = 28$) and patients with *STAT1* LOF mutations ($n = 6$) are indicated. All differences between healthy controls and patients with *STAT1* LOF alleles were not significant. (F) Secretion of IL-12p70 by whole blood cells, as determined by ELISA, in the absence of stimulation (open symbols), after stimulation with BCG (lightly colored symbols), or BCG + IFN-γ for 48 h (closed symbols). Horizontal bars represent medians. The p-values for differences between patients with *STAT1* GOF mutations ($n = 15$) and controls ($n = 23$) and patients with *STAT1* LOF mutations ($n = 6$) are indicated and were calculated in nonparametric Wilcoxon tests. All experiments were performed at least two times independently.

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The mutant *STAT1* alleles described herein enhance cellular responses to cytokines such as IFN-α/β, IFN-γ, and IL-27, which potently inhibit the development of IL-17–producing T cells via STAT1 (Batten et al., 2006; Yoshimura et al., 2006; Stumhofer et al., 2006; Amadi-Obi et al., 2007; Feng et al., 2008; Kimura et al., 2008; Tanaka et al., 2008; Chen et al., 2009; Ramgolam et al., 2009; Crabé et al., 2009; Diveu et al., 2009; El-behi et al., 2009; Guzzo et al., 2010; Villarino et al., 2010; Liu and Rohowsky-Kochan, 2011). These mutant alleles also increase cellular responses to IL-6 and IL-21, which normally induce IL-17–producing T cells via STAT3 rather than STAT1 (Hirahara et al., 2010). Enhanced STAT1-dependent cellular responses to these two groups of cytokines probably impair the development of IL-17–producing T cells. It remains unclear whether this mechanism predominantly involves IL-17–inhibiting cytokines (IFN-α/β, IFN-γ, and IL-27), either individually or in combination. The available data from the mouse model suggest that IL-27 is the most potent of the three inhibitors. There is also evidence that these cytokines inhibit IL-17–producing T cell development in humans (Ramgolam et al., 2009; Liu and Rohowsky-Kochan, 2011). Enhanced STAT1 and GAF activation in response to the IL-17 inducers IL-6 and IL-21, and perhaps IL-23, may also play a key role in disease, by antagonizing STAT3 responses. The effect of the aryl hydrocarbon receptor on IL-17 T cell development might also be enhanced by gain-of-function *STAT1* alleles (Kimura et al., 2008). Moreover, enhanced STAT1 activity downstream from IL-22 and IL-26 in cells, not detected in our study, might also contribute to the CMCD phenotype. Finally, thyroid autoimmunity in eight patients and systemic lupus erythematosus in another patient in our series probably resulted from the enhancement of IFN-α/β responses, as such autoimmunity is a frequent adverse effect of treatment with recombinant IFN-α or IFN-β (Oppenheim et al., 2004; Selmi et al., 2006). Importantly, no autoantibodies against IL-17A, IL-17F, or IL-22 were detected in the patients' serum (Table I and unpublished data).

Remarkably, germline mutations in human *STAT1* underlie susceptibility to three different types of infectious disease: mycobacterial diseases, viral diseases, and CMC. Patients bearing *STAT1* mutations and displaying mycobacterial and/or viral disease do not suffer from CMC, and the patients with CMCD caused by other *STAT1* alleles described here present no mycobacterial or viral disease. The pathogenic mechanisms involved are clearly different, with loss-of-function mutations in *STAT1* underlying mycobacterial and viral diseases (Dupuis et al., 2001, 2003; Chapgier et al., 2006b, 2009; Kong et al., 2010; Averbuch et al., 2011; Kristensen et al., 2011). Human AR STAT1 deficiency impairs cellular responses to IFN-α/β, IFN-γ, IFN-λ, and IL-27 (Dupuis et al., 2003; Chapgier et al., 2006b, 2009; Kong et al., 2010; Kristensen et al., 2011). Viral diseases probably result from impaired IFN-α/β and, perhaps, IFN-λ immunity, although impaired IFN-γ and IL-27 immunity may also contribute to the phenotype. Patients with AD MSMD, heterozygous for loss-of-function dominant-negative mutations of *STAT1,* suffer from mycobacterial disease caused by the impairment of IFN-γ immunity (Chapgier et al., 2006a; Dupuis et al., 2001). Overall, mutations impairing STAT1 function confer AD or AR susceptibility to intracellular agents, through the impairment of IFN-α/β (viral diseases) and/or IFN-γ immunity (mycobacterial diseases). In contrast, the gain-of-function *STAT1* mutations reported here confer AD CMCD because of the enhancement of STAT1-mediated cellular responses to STAT1-dependent repressors and STAT3-dependent inducers of IL-17–producing T cells. These studies neatly demonstrate that severe infectious diseases in otherwise healthy patients may be subject to genetic determinism (Casanova and Abel, 2005, 2007; Alcaïs et al., 2009, 2010). They also highlight the profoundly different effects that germline mutations in the same human gene may have, resulting in different infectious diseases through different molecular and cellular mechanisms.

MATERIALS AND METHODS

Massively parallel sequencing

DNA (3 µg) extracted from EBV-B cells from the patient was sheared with a S2 Ultrasonicator (Covaris). An adapter-ligated library was prepared with the Paired-End Genomic DNA Sample Prep kit (Illumina). The SureSelect Human All Exon kit (Agilent Technologies) was then used for exome capture. Single-end sequencing was performed on a Genome Analyzer IIx (Illumina), generating 72-base reads.

Sequence alignment, variant calling, and annotation

BWA aligner (Li and Durbin, 2009) was used to align the sequences obtained with the human genome reference sequence (hg18 build). Downstream processing was performed with the Genome analysis toolkit (GATK; McKenna et al., 2010), SAMtools (Li et al., 2009), and Picard Tools (http://picard.sourceforge.net). Substitution calls were made with a GATK UnifiedGenotyper, whereas indel calls were made with a GATK IndelGenotyperV2. All calls with a read coverage ≤2x and a Phred-scaled SNP quality of ≤20 were filtered out. All the variants were annotated with annotation software that was developed in-house. The data were further analyzed with sequence analysis software that had been developed in-house (SQL database query–driven system).

Molecular genetics

EBV-B cells and the STAT1-deficient cell line U3C were cultured as previously described (Chapgier et al., 2006a). Primary fibroblasts were cultured in DME supplemented with 10% fetal calf serum. Cells were stimulated with the indicated doses (in IU/ml or ng/ml) of IFN-γ (Imukin; Boehringer Ingelheim), IFN-α2b (IntronA; Schering-Plough), IL-27 (R&D Systems), IL-21 (R&D Systems), IL-22 (R&D Systems), IL-23 (R&D Systems), and IL-6 (R&D Systems). Genomic DNA and total RNA were extracted from cell lines and fresh blood cells, as previously described (Chapgier et al., 2006a). Genomic DNA was amplified with specific primers encompassing exons 6–10 of *STAT1* (available upon request), sequenced with the Big Dye Terminator cycle sequencing kit (Applied Biosystems), and analyzed on an ABI Prism 3730 (Applied Biosystems). We used the various alleles of *STAT1* in the pcDNA3 *STAT1*-V5 vector (Chapgier et al., 2006a; Kong et al., 2010). We generated the various *STAT1* mutations by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis kit; Stratagene) with the mismatched primers listed in Table S4. U3C cells were harvested by trypsin treatment 24 h before transfection and replated at a density of 2.5×10^5 cells/ml in 6-well plates. Plasmid DNA (5 µg per plate) carrying the WT or all the various mutant *STAT1* alleles was used for cell transfection with the Calcium Phosphate Transfection kit (Invitrogen).

Luciferase reporter assay

U3C cells were dispensed into 96-well plates (1×10^4/well) and transfected with reporter plasmids (Cignal GAS and ISRE Reporter Assay kit;

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SABiosciences) and plasmids carrying the various alleles of *STAT1* or a mock vector, in the presence of Lipofectamine LTX (Invitrogen). 6 h after transfection, the cells were transferred back into medium containing 10% FBS and cultured for 24 h. The transfectants were then stimulated with IFN-γ (500 and 1,000 IU/ml), IL-27 (20 and 100 ng/ml), and IFN-α (500, 1,000, and 5,000 IU/ml) for 16 h and subjected to luciferase assays with the Dual-Glo luciferase assay system (Promega). Experiments were performed in triplicate and firefly luciferase activity was normalized with respect to *Renilla* luciferase activity. The data are expressed as fold induction with respect to nonstimulated cells.

Immunoblot analysis and electrophoretic mobility shift assays

The following optimal stimulation conditions were used. EBV-B or U3C cells were stimulated by incubation for 20 min with 100 μg/ml IL-21 or 25 ng of IL-22; 30 min with 10^3 or 10^5 IU/ml IFN-γ and IFN-α; 15 min with 50 ng/ml IL-6; or 30 min with 50 or 100 ng/ml IL-27. WB was performed as previously described (Dupuis et al., 2003). In brief, cell activation was blocked with cold 1X PBS, cells were lysed in 1% NP-40 lysis buffer, and the proteins were recovered and subjected to SDS-PAGE. We used antibodies directed against phosphorylated STAT1 (pY701; BD), STAT1 (C-24; Santa Cruz Biotechnology), V5 (Invitrogen), α-tubulin (Santa Cruz Biotechnology), phosphorylated STAT3 (Cell Signaling Technology), lamin B1 (Santa Cruz Biotechnology), GAPDH (Santa Cruz Biotechnology), and STAT3 (Santa Cruz Biotechnology). EMSA was performed as previously described (Chapgier et al., 2006a). In brief, cell activation was blocked by incubation with cold 1X PBS, and the cells were gently lysed to remove cytoplasmic proteins while keeping the nucleus intact. We then added nuclear lysis buffer and recovered the nuclear proteins, which were subjected to nondenaturing electrophoresis with radiolabeled GAS (from the FCγR1 promoter: 5′-ATGTATTTCCCAGAAA-3′) and ISRE (from the ISG15 promoter: 5′-GATCGGGAAAGGGAAACCGAAACTGAA-3′) probes.

Staurosporine and pervanadate treatment of cells

We assessed dephosphorylation by stimulating U3C transfectants with 10^5 IU/ml IFN-γ. The cells were then washed and incubated with 1 μM staurosporine in DME for 15, 30, or 60 min. The cells were then lysed with 1% NP-40 lysis buffer, and the proteins recovered were subjected to immunoblot analysis.

Pervanadate was prepared by mixing orthovanadate with H_2O_2 for 15 min at 22°C. U3C transfectants were treated with pervanadate (0.8 mM orthovanadate and 0.2 mM H_2O_2) 5 min before stimulation. They were then stimulated with IFN-γ for 20 min. The stimulation was stopped by adding cold 1X PBS. The proteins were recovered and subjected to immunoblot analysis.

Extraction of nuclear and cytoplasmic proteins

U3C transfectants or EBV-B cells were stimulated with IFN-γ or IFN-α for 20 min and subjected to nuclear and cytoplasmic protein extraction with NE-PER Nuclear and Cytoplasmic Extraction Regents (Thermo Fisher Scientific) according to the manufacturer's protocol.

Immunofluorescence staining

Immunofluorescence experiments were performed as previously described (Chapgier et al., 2006a). In brief, cells (transfected U3C or SV-40 fibroblasts) were stimulated for the times indicated with 10,000 IU/ml of IFN-γ. Cells were then washed with cold PBS and fixed with 4% PFA. The cells were washed and incubated with an antibody against STAT1, which was then detected by incubation with an Alexa Fluor 488–conjugated anti–mouse antibody.

T cell blast differentiation and stimulation

PBMCs were recovered by centrifuging blood samples on Ficoll gradients, as previously described (Chapgier et al., 2006a). They were then cultivated, at a density of 1 million cells per ml in RPMI supplemented with 10% fetal calf serum and stimulated with phytohemagglutinin (1 μg/ml) for 3 d. Cells were then recovered, centrifuged on a Ficoll gradient, cultivated (at a density of 0.2 million cells/ml) to Panserin 401 supplemented with 10% FCS and glutamine 1X, and stimulated with 40 IU/ml IL-2 (Roche). Cells were then incubated for 30 min with 100 ng/ml IL-23. Activation was stopped by adding 1X cold PBS, and cells were processed for immunoblot analysis.

Modeling

Images of the three-dimensional structure of STAT1 (Chen et al., 1998) were generated with the 2002 PyMOL Molecular Graphics System (DeLano Scientific), using PDB accession no. 1BF5.

Whole-blood assay of the IL-12–IFN-γ circuit

Whole-blood assays were performed as previously described (Feinberg et al., 2004). Heparin-treated blood samples from healthy controls and patients were stimulated in vitro with live *Mycobacterium bovis* BCG (Pasteur) alone or with IFN-γ (5,000 IU/ml; Boehringer Ingelheim). Supernatants were collected after 48 h of stimulation, and ELISA were performed with specific antibodies directed against IL-12p40 or IL-12p70, using kits from R&D Systems according to the manufacturer's instructions.

Production of IL-17A, IL-17F, and IL-22 by leukocytes

Cell activation. IL-17A– and IL-22–producing T cells were evaluated by intracellular staining or by ELISA, as previously described (de Beaucoudrey et al., 2008). In brief, PBMCs were purified by centrifugation on a gradient (Ficoll-Paque PLUS; GE Healthcare) and resuspended in RPMI supplemented with 10% FBS (RPMI/10% FBS; Invitrogen). Adherent monocytes were removed from the PBMC preparation by incubation for 2 h at 37°C, under an atmosphere containing 5% CO_2.

For ex vivo evaluation of IL-17– and IL-22–producing T cells by flow cytometry, we resuspended 5×10^6 nonadherent cells in 5 ml RPMI/10% FBS in 25 cm^2 flasks and stimulated them by incubation with 40 ng/ml PMA (Sigma-Aldrich) and 10^{-5} M ionomycin (Sigma-Aldrich) in the presence of a secretion inhibitor (1 μl/ml GolgiPlug; BD) for 12 h.

For evaluation of the IL-17– and IL-22–producing T cell blasts after in vitro differentiation, the nonadherent PBMCs were dispensed into 24-well plates at a density of 2.5×10^6 cells/ml in RPMI/10% FBS and activated with 2 μg/ml of an antibody directed against CD3 (Orthoclone OKT3; Janssen-Cilag) alone, or together with 5 ng/ml TGF-β1 (240-B; R&D Systems), 20 ng/ml IL-23 (1290-IL; R&D Systems), 50 ng/ml IL-6 (206-IL; R&D Systems), 10 ng/ml IL-1β (201-LB; R&D Systems), or combinations of these four cytokines. After 3 d, the cells were restimulated in the same activation conditions, except that the anti-CD3 antibody was replaced with 40 IU/ml IL-2 (Proleukin i.v.; Chiron). We added 1 ml of the appropriate medium, resuspended the cells by gentle pipetting, and then split the cell suspension from each well into two. Flow cytometry was performed on one of the duplicated wells 2 d later, after stimulation by incubation for 12 h with 40 ng/ml PMA and 10^{-5} M ionomycin in the presence of 1 μl/ml GolgiPlug. FACS analysis was performed as described in the following section. The other duplicated well was split into two, with one half left unstimulated and the other stimulated by incubation with 40 ng/ml PMA and 10^{-5} M ionomycin for another 2 d. Supernatants were collected after 48 h of incubation, for ELISA.

Flow cytometry. Cells were washed in cold PBS, and surface labeling was achieved by incubating the cells with PECy5-conjugated anti–human CD3 antibody (BD) in PBS/2% FBS for 20 min on ice. Cells were then washed twice with 2% FBS in cold PBS, fixed by incubation with 100 μl of BD Cytofix for 30 min on ice, and washed twice with BD Cytoperm (Cytofix/Cytoperm Plus, fixation/permeabilization kit; BD). Cells were then incubated for 1 h on ice with Alexa Fluor 488–conjugated anti–human IL-17A (53–7179-42; eBioscience), PE-conjugated anti–human IL-22 (IC7821P; R&D Systems), or PE-conjugated anti–human IFN-γ (IC285P; R&D Systems) antibodies, washed twice with Cytoperm, and analyzed with a FACSCanto II system (BD).

ELISA. IL-17A, IL-17F, and IL-22 levels were determined by ELISA on the supernatants harvested after 48 h of whole-blood stimulation with 40 ng/ml PMA and 10^{-5} M ionomycin, or after in vitro PHA blast differentiation and

48 h of stimulation with 40 ng/ml PMA and 10^{-5} M ionomycin. We used anti–human IL-17A and anti–human IL-22 Duoset kits (R&D Systems) and the anti–human IL-17F ELISA Ready-SET-GO! set (eBioscience).

Statistical analysis. We assessed differences between controls, MSMD patients bearing loss-of-function *STAT1* alleles, and CMCD patients bearing gain-of-function *STAT1* alleles in terms of the percentages of IL-17A– and IL-22–producing T cells, as assessed by flow cytometry, and in terms of the amounts of IL-17A, IL-17F, and IL-22 produced in various stimulation conditions, as assessed by ELISA. We used the nonparametric Wilcoxon test, as implemented in the PROC NPAR1WAY of the SAS software version 9.1 (SAS Institute). For all analyses, $P < 0.05$ was considered statistically significant.

Online supplemental material

Fig. S1 shows that STAT1-CMCD mutants are gain-of-function alleles by loss of nuclear dephosphorylation. Fig. S2 is a schematic representation of the cytokines and transcription factors directing the development of naive CD4 cells into IL-17–producing T cells. Fig. S3 shows the normal response of CMCD patient cells to IFN-α in terms of ISGF3 activation, to IFN-γ in terms of STAT1 nuclear translocation; and to IL-23 and IL-22 in terms of pSTAT3. Fig. S4 shows impaired in vitro differntiation of IL-17– and Il-22–producing T cell blasts in patients with CMCD and gain-of-function SATA1 mustations. Table S1 shows novel coding heterozygous variants found by whole-exome sequencing in the 6 different patients. Table S2 shows novel coding heterozygous variants found by whole-exome sequencing within genes shared by more than one patient. Table S3 lists conservation and predictions on the function of the mutant STAT1 alleles associated with CMCD. Table S4 lists the STAT1 GOF mutation created, and the pair of primers used. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110958/DC1.

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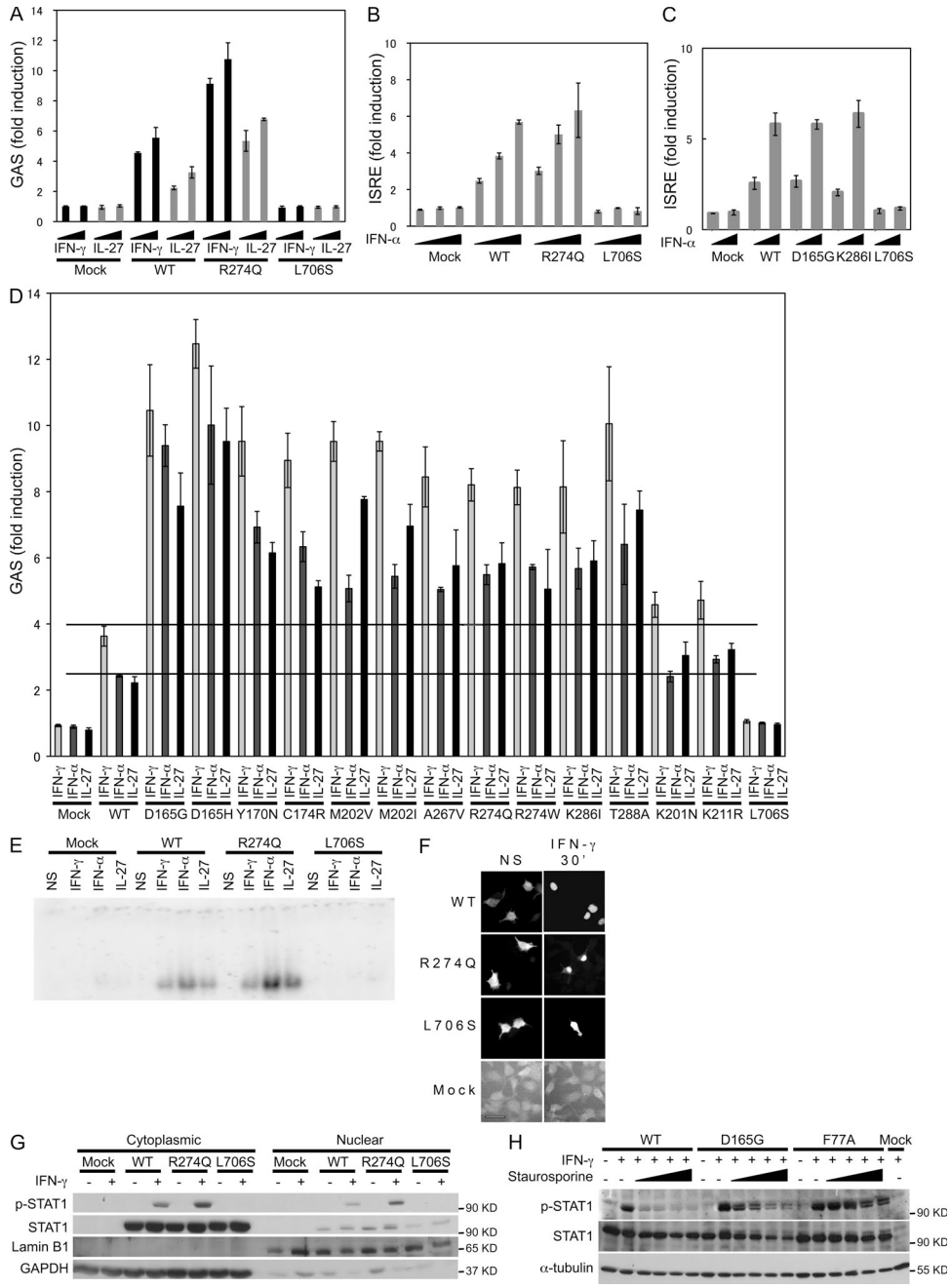

Figure S1. STAT1-CMCD mutants are gain-of-function alleles by loss of nuclear dephosphorylation. The response to various doses of IFN-γ, IFN-α or IL-27 (A-C) was evaluated by determining luciferase activity of reporter genes under the control of the GAS promoter (A) and the ISRE promoter (B and C), in U3C cells transfected with a mock vector, a WT form, or the mutant forms (R274Q, D165G, K286I and L706S) of STAT1. Experiments were performed independently at least three times. (D) The response to IFN-γ, IFN-α, and IL-27 was evaluated by determining luciferase activity of a reporter gene under the control of the GAS promoter in U3C cells transfected with a mock vector, a WT allele of *STAT1*, or 11 CMCD-causing *STAT1* alleles (D165G, D165H, Y170N, C174R, M202V, M202I, A267V, R274Q, R274W, K286I, and T288A), as well as the known K201N, K211R, and L706S *STAT1* alleles. The two horizontal lanes show the response of the WT *STAT1* allele to cytokine stimulation. The experiment was performed twice. (E) GAF-DNA-binding activity in U3C cells transfected with mock, WT, R274Q, and L706S alleles of *STAT1* ; left unstimulated (NS); or stimulated with IFN-γ, IFN-α, or IL-27; the results shown are representative of at least two independent experiments. (F) Immunofluorescence of U3C cells transfected with WT, R274Q, and L706S alleles of *STAT1* without (NS) and with IFN-γ stimulation and stained with an antibody specific for STAT1. Bar, 50 µm. The pictures shown are representative of the cells observed. (G) The cytoplasmic (visualized by the GAPDH antibody) and nuclear (visualized by the Lamin B1 antibody) fractions of U3C cells transfected with mock, WT, R274Q, and L706S alleles of *STAT1*, with and without IFN-γ stimulation, were tested for the presence of total and phosphorylated STAT1 by WB. We loaded the equivalent of 25 µg of protein for the cytoplasmic fraction and 8 µg of protein for the nuclear fraction. The experiment was performed twice. (H) The nuclear dephosphorylation of STAT1 was assessed in U3C cells transfected with a mock vector, a WT *STAT1* allele, the D165G, and the F77A *STAT1* mutant alleles (the latter being known to impair STAT1 dephosphorylation) after treatment with IFN-γ and the tyrosine kinase inhibitor staurosporine for increasing periods of time (30, 60, 90, and 120 min); the results shown are representative of at least two independent experiments.

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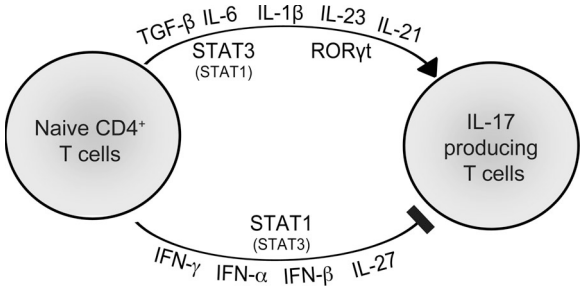

Figure S2. Schematic representation of the cytokines and transcription factors directing the development of naive CD4 T cells into IL-17–producing T cells. Activating molecules, such as IL-6, IL-1β, IL-23, and IL-21 (acting mostly through STAT3, RORγt, and, to a lesser extent, STAT1), TGF-β, and inhibiting molecules, such as IFN-γ, IFN-β, IFN-α, and IL-27 (acting mostly through STAT1 and, to a lesser extent, STAT3) are represented.

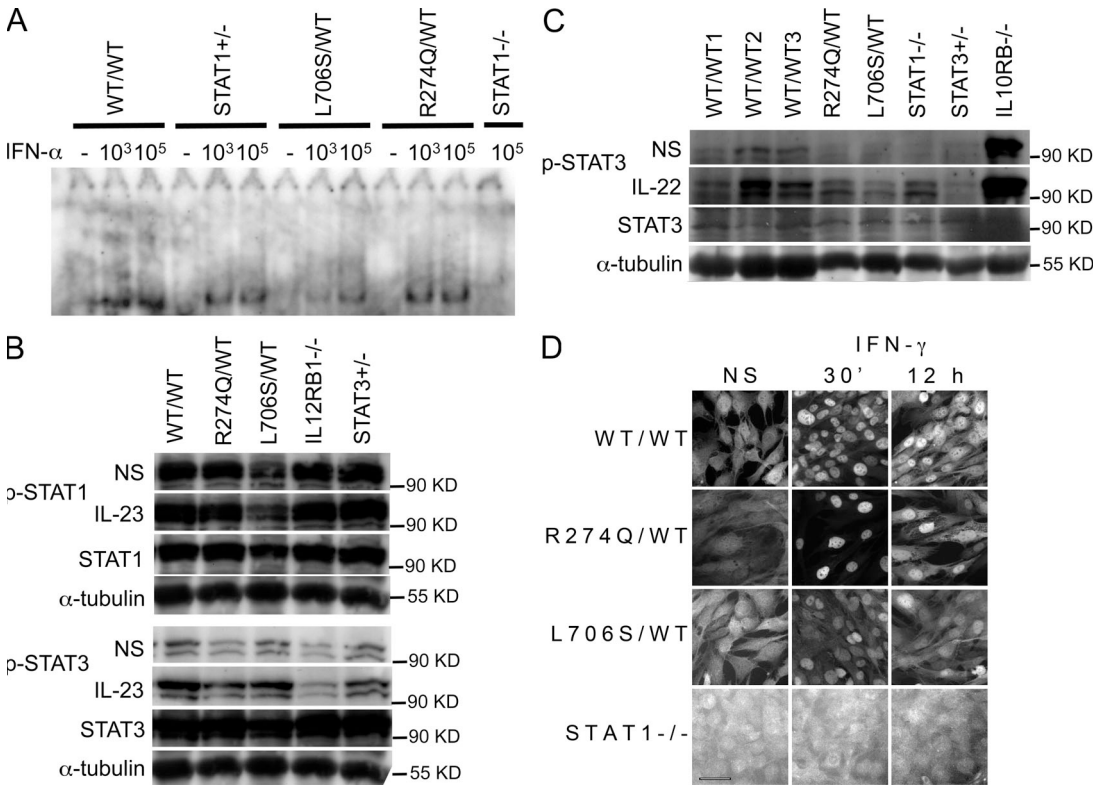

Figure S3. Normal response of CMCD patient cells to IFN-α in terms of ISGF3 activation; to IFN-γ in terms of STA1 nuclear translocation; and to IL-23 and IL-22 in terms of pSTAT3. (A) The response of the (R274Q/WT) patient's EBV-B cells was evaluated by EMSA with an ISRE probe and was compared to those of a healthy control (WT/WT), heterozygous cells with a WT and a loss-of-function allele (STAT1$^{+/-}$), cells heterozygous for a dominant loss-of-function mutation of *STAT1* (L706S/WT), and cells with complete STAT1 deficiency (STAT1$^{-/-}$). Cells were stimulated with various doses of IFN-α (international unit/milliliter); the results shown are representative of at least two independent experiments. (B) The response to IL-23 of T cell blasts was evaluated in control (WT/WT), CMCD (R274Q/WT), MSMD (L706S/WT), IL12RB1-deficient (IL12RB1$^{-/-}$) and heterozygous STAT3 (STAT3$^{+/-}$) cells by WB. The experiment was performed twice. (C) The response to IL-22 of primary fibroblasts was evaluated in three controls (WT/WT1, 2, and 3), CMCD (R274Q/WT), MSMD (L706S/WT), STAT1-deficient (STAT1$^{-/-}$), heterozygous STAT3 (STAT3$^{+/-}$), and IL10RB-deficient (IL10RB$^{-/-}$) cells by WB; the results shown are representative of at least two independent experiments. (D) Immunofluorescence for STAT1 of SV-40–transformed fibroblasts with and without IFN-γ stimulation, for a control (WT/WT), a CMCD patient (R274Q/WT), an MSMD patient (L706S/WT), and a complete STAT1-deficient patient (STAT1$^{-/-}$). Bar, 50 μm. The results shown are representative of at least two independent experiments

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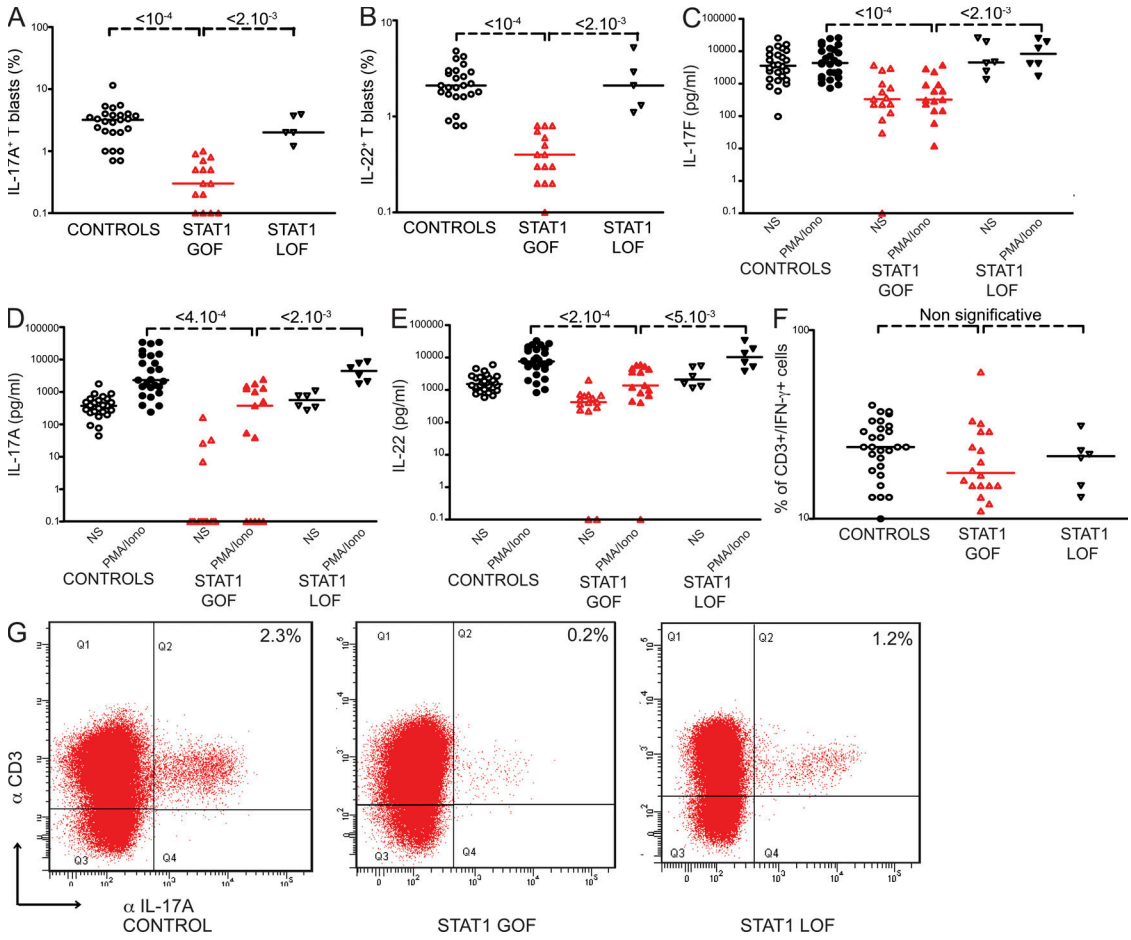

Figure S4. Impaired in vitro differentiation of IL-17– and IL-22–producing T cells in patients with AD CMCD and *STAT1* mutations. Each symbol represents an individual control (black circles), a patient with a *STAT1* GOF mutation (red triangles), or a patient with one or two *STAT1* LOF mutations (black upside-down triangles). The results shown are representative of at least two independent experiments. (A and B) IL-17$^+$ (A) and IL-22$^+$ (B) T cell blasts were expanded in vitro in presence of anti-CD3 antibody, IL-2, IL-1β, and IL-6 for 5 d, followed by 12 h of stimulation with PMA and ionomycin. C- E. Secretion of IL-17F (C), IL-17A (D), and IL-22 (E) by T cell blasts expanded in vitro in presence of anti-CD3 antibody, IL-2, IL-1β, and IL-6 for 5 d, followed by 12 h of stimulation with PMA and ionomycin. Horizontal bars represent medians. The p-values for the nonparametric Wilcoxon test, between patients with *STAT1* GOF mutations (n = 18) and healthy controls (n = 28) and patients with *STAT1* LOF mutations (n = 6) are indicated. All differences between healthy controls and patients with *STAT1* LOF alleles were nonsignificant. (F) Percentage of CD3$^+$/IFN-γ$^+$ cells, as determined by flow cytometry, in nonadherent PBMCs activated by incubation for 12 h with PMA and ionomycin. Horizontal bars represent medians. The p-values for differences between patients with *STAT1* GOF mutations (n = 18) and healthy controls (n = 28) and patients with *STAT1* LOF mutations (n = 6) were calculated in nonparametric Wilcoxon tests and were nonsignificant. (G) Flow cytometry analysis of CD3 and IL-17A in nonadherent PBMCs activated with PMA-ionomycin, from a control (left), a STAT1 GOF patient (middle), and a STAT1 LOF patient (right). The percentage of CD3$^+$/IL17A$^+$ cells is indicated in the top right corner of each dot plot.

Table S1, which shows all novel coding heterozygous variants found by whole exome sequencing in six different patients, is available as an excel file.

Table S2, which shows all novel coding heterozygous variants found by whole-exome sequencing within genes shared by more than one patient, is available as an excel file.

Table S3. Conservation and predictions on the function of the mutant *STAT1* alleles associated with CMCD

Mutation	Polyphen II score	Damaging	Conservation
D165G	0.247	Possibly	Poor (E, N, Y found at this position)
D165H	0.469	Possibly	
Y170N	0.819	Possibly	Poor (R, H, F found at this position)
C174R	0.000	Benign	Very Poor (R found in two fishes, plus H, F, I, E Y, N, M, K)
M202V	0.794	Possibly	High (V found in the fish)
M202I	0.956	Probably	
A267V	0.998	Probably	High (G and I found at this position)
Q271P	0.932	Possibly	High (F and L found at this position)
R274W	1.000	Probably	Very High (no variation found at this position)
R274Q			
K286I	0.961	Probably	Very High (no variation found at this position)
T288A	0.997	Probably	High (S found at this position in the fish)

Summary of the Polyphen II score, possible functional consequences (possibly, probably damaging, or benign), and the conservation of the amino acid for the species sequenced for STAT1.

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Table S4. Primers used for each STAT1 GOF mutation

Mutation	Primers (Forward+Reverse)
D165G	5’-AGA GCC TGG AAG GTT TAC AAG ATG A-3’ 5’-TCA TCT TGT AAA CCT TCC AGG CTC T-3’
D165H	5’-AAG AGC CTG GAA CAT TTA CAA GAT G-3’ 5’-CAT CTT GTA AAT GTT CCA GGC TCT T-3’
Y170N	5’-TTA CAA GAT GAA AAT GAC TTC AAA T-3’ 5’-ATT TGA AGT CAT TTT CAT CTT GTA A-3’
C174R	5’-TAT GAC TTC AAA CGC AAA ACC TTG C-3’ 5’-GCA AGG TTT TGC GTT TGA AGT CAT A-3’
M202I	5’-ACT CAA GAA GAT ATA TTT AAT GCT T-3’ 5’-AAG CAT TAA ATA TAT CTT CTT GAG T-3’
M202V	5’-TTA CTC AAG AAG GTG TAT TTA ATG C-3’ 5’-GCA TTA AAT ACA CCT TCT TGA GTA A-3’
A267V	5’-TCA CTA TAG TTG TGG AGA GTC TGC A-3’ 5’-TGC AGA CTC TCC ACA ACT ATA GTG A-3’
R274Q	5’-TGC AGC AAG TTC AGC AGC AGC TTA A-3’ 5’-TTA AGC TGC TGC TGA ACT TGC TGC A-3’
R274W	5’-CTG CAG CAA GTT TGG CAG CAG CTT A-3’ 5’-TAA GCT GCT GCC AAA CTT GCT GCA G-3’
K286I	5’-AAT TGG AAC AGA TAT ACA CCT ACG A-3’ 5’-TCG TAG GTG TAT ATC TGT TCC AAT T-3’
T288A	5’-GAA CAG AAA TAC GCC TAC GAA CAT G-3’ 5’-CAT GTT CGT AGG CGT ATT TCT GTT C-3’
K211R	5’-ACA ATA AGA GAA GGG AAG TAG TTC A-3’ 5’-TGA ACT ACT TCC CTT CTC TTA TTG T-3’

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ANNEX

Publication 3

Gain-of-function STAT1 mutations underlying CMC: enhanced responses to IFN-α/β, IFN-γ, and IL-27 impair IL-17 T-cell immunity

Okada S*,Cypowyj S*,**Liu L***, Kong XF*, Kreins AY, Mekki N,Toubiana J,Hiller J,Okada C, Boisson B, Morel JD, Soltész B, Tóth B, Bensifi M, Toulon A, Gulácsy V, Schnopp C, Schaller M, Wijaya-Kusuma T, Jansson A, Sawalle J, Marie-Cardine A, Bue M, Drexel B, Hoernes M, Bustamante J, Firinu D, Meuwissen H, Grimprel E, Karmochkine M, Weiss L, Becerra J, Lagos M, Fouyssac F, Wallace MR, Lortholary O, Tucker M, Willis M, Leonard S, Ferdman R, Church J, Fieschi C, Suarez F, Hermine O, Kerns L, Yuenyongviwat A, Polak M, Bodemer C, Reich K, Debre M, Belohradsky B, Dupont B, Roesler J, Bousfiha A, Sanal O, Fischer A, Blanche S, Muhsen S, Kobayashi M, Reichenbach J, Seger R, Klein C, Renner E, Abel L, Traidl-Hoffmann C, Picard C, Maródi L, Boisson-Dupuis S*, Puel A* and Casanova JD*

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submitted

Gain-of-function *STAT1* mutations underlying CMC:

enhanced responses to IFN-α/β, IFN-γ, and IL-27 impair IL-17 T-cell immunity

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Abstract

Autosomal dominant (AD) chronic mucocutaneous candidiasis disease (CMCD) may be caused by heterozygous mutations affecting the coiled-coil domain (CCD) of human STAT1. These mutations are gain-of-function due to a loss of dephosphorylation, resulting in enhanced STAT1 activity in response to all cytokines tested. These mutations also impair the development of IL-17-producing T cells from bulk leukocytes, both *ex vivo* and *in vitro*, potentially accounting for AD CMCD. We report here a new set of AD CMCD-causing mutations affecting the DNA-binding domain (DBD) of STAT1 yet impairing the nuclear dephosphorylation of activated STAT1, resulting in a gain of function. We also found that CMCD patients heterozygous for CCD or DBD gain-of-function *STAT1* alleles displayed poor IL-17-producing T-cell development from naïve $CD4^+$ T cells *in vitro*. Finally, we report that the STAT1-dependent cytokines IFN-α/β, IFN-γ and IL-27 further inhibit the development of IL-17 T cells from the naïve CD4 T cells of these CMCD patients, whereas antibodies against these cytokines can rescue the development of IL-17 T cells. In conclusion, gain-of-function mutations affecting the CCD and DBD of STAT1 cause AD CMCD by the same mechanism: impaired STAT1 nuclear dephosphorylation, enhanced cellular responses to IFN-α/β, IFN-γ and IL-27, and the impaired development of IL-17 T cells.

Introduction

Chronic mucocutaneous candidiasis disease (CMCD) is typically defined as infections of the nails, skin, oral and genital mucosae with *Candida albicans* in patients with no other prominent clinical signs (Canales *et al*., 1969; Chilgren *et al.,* 1967; Kirkpatrick, 2001; Leroy *et al.,* 1989; Lilic, 2002; Wells *et al*., 1972; Williamson, 1969). This definition is not strict, as some CMCD patients display other infectious diseases, of a bacterial or fungal nature in particular, or autoimmune signs, such as hypothyroidism and even systemic lupus erythematosus. They may also present mucocutaneous carcinomas and cerebral aneurysms. The term CMCD is best used to refer to patients with CMC displaying none of the known primary immunodeficiency diseases (PIDs) associated with a predisposition to CMC, such as profound T-cell deficits (Puel *et al*., 2012), autosomal recessive (AR) CARD9 deficiency (Glocker *et al*., 2009), autosomal dominant (AD) hyper IgE syndrome (AD-HIES) (Minegishi, 2009), AR autoimmune polyendocrinopathy syndrome type 1 (APS-1) (Kisand *et al*., 2011) and AR IL-12Rβ1 or IL-12p40 deficiency (de Beaucoudrey *et al*., 2010; Ouederni *et al*., in preparation; Prando *et al*., 2013). CMCD was first described clinically in 1969, but it was not until 2011 that the first genetic etiologies of CMCD were deciphered, with the identification of AR IL-17RA and AD IL-17F deficiencies, in one family each (Puel *et al*., 2011). This candidate gene approach was based on the previous discovery of impaired IL-17 T-cell development in patients with AD HIES (de Beaucoudrey *et al*., 2008; Ma *et al*., 2008; Milner *et al*., 2008; Minegishi *et al*., 2009; Puel *et al*., 2010b; Renner *et al*., 2008) and high titers of circulating and neutralizing autoantibodies against IL-17 cytokines in patients with AR APS-1 (Kisand *et al*., 2010; Puel *et al*., 2010a; Puel *et al*., 2010b).

Collectively, these data indicated that CMCD may result from inborn errors of IL-17 immunity (Puel *et al*., 2010b). Going beyond these rare conditions, these data also suggested that CMC results from impaired IL-17 immunity in patients with various inherited or acquired conditions, such as AIDs patients and patients on immunosuppressive treatments (Puel *et al*., 2012; Puel *et al*., 2010b).

Surprisingly, genome-wide approaches then led to the discovery of 14 heterozygous mutations, all clustered in the region encoding the coiled-coil domain (CCD) of STAT1, in 78 patients from 33 kindreds with CMCD (Hori *et al*., 2012; Liu *et al*., 2011; Smeekens *et al.,* 2011; Takezaki *et al*., 2012; Toth *et al*., 2012; van de Veerdonk *et al*., 2011; Wang *et al*., 2012; Romberg *et al*, 2013). Two heterozygous mutations affecting the DNA-binding domain (DBD) of STAT1 (T385M and E353K) have also been reported in three patients from three unrelated kindreds (Romberg *et al*., 2013; Takezaki *et al*., 2012). Most of these CMCD-causing *STAT1* mutations were shown to be gain-of-function (GOF) due to a loss of nuclear dephosphorylation (Liu *et al*., 2011; Takezaki *et al*., 2012). Consistent with these findings, cells heterozygous for these mutations displayed enhanced STAT1 activity in response to cytokines, including IFN-α/β, IFN-γ and IL-27, which activate predominantly STAT1 rather than STAT3, and in response to cytokines such as IL-6 and IL-21, which normally predominantly activate STAT3 rather than STAT1 (Liu *et al.,* 2011). These patients also displayed poor IL-17 T-cell development from bulk leukocytes both *ex vivo* and *in vitro* (Liu *et al*., 2011; Romberg *et al*., 2013; Smeekens *et al*., 2012; Takezaki *et al*., 2012; van de Veerdonk *et al*., 2011). This deficit could be accounted for by enhanced cellular responses to STAT1-dependent repressors of IL-17-producing T cells, such as IFN-α/β IFN-γ and IL-27, as

inferred from studies in mice (Batten *et al.,* 2006; Diveu *et al*., 2009; El-behi *et al*., 2009; Feng *et al*., 2008; Hirahara *et al*., 2012; Stumhofer *et al*., 2006; Villarino *et al.,* 2010; Yoshimura *et al*., 2006) and humans (Amadi-Obi *et al*., 2007; Chen *et al*., 2009; Crabe *et al*., 2009; Guzzo *et al*., 2010; Liu & Rohowsky-Kochan, 2011; Ramgolam *et al*., 2009). Alternatively, it may be due to the enhanced and potentially competing STAT1 responses observed after stimulation with STAT3-dependent inducers of IL-17, such as IL-6, IL-21 and IL-23, in mice (Bettelli *et al*., 2006; Zhou *et al*., 2007) and humans (Kishimoto, 2005; Sallusto *et al*., 2012). We report here the identification of 58 additional CMCD patients with GOF mutations affecting the CCD (28 patients from 20 kindreds) and DBD (30 patients from 18 kindreds) of STAT1. We also show that both CCD and DBD mutations inhibit the development of IL-17 T cells from naïve $CD4^+$ T cells *in vitro*. Finally, we show that the mechanism underlying the poor development of IL-17 T cells, resulting in CMC, involves enhanced responses to IFN-α/β, IFN-γ and IL-27.

Results

We sequenced the complete coding exons and flanking intron regions of *STAT1* in 130 patients suffering from sporadic or familial CMCD. We found known heterozygous missense mutations affecting the CCD of STAT1 in 19 patients from 13 new families (Fig. 1, Table 1). We also identified seven other kindreds (nine patients), each with a previously unknown missense mutation affecting the CCD. Finally, we identified heterozygous missense mutations affecting the DBD of STAT1 — two (T385M, E353K) already reported (Takezaki *et al*., 2012; Romberg *et al.,* 2013) and 10 previously unknown — in 30 patients from 18 families (Fig. 1, Table 1). The intrafamilial

segregation of the mutations was consistent with an AD trait, and clinical penetrance appeared to be complete, as all patients with CMCD from the kindreds tested were heterozygous, whereas none of these mutations was found in the heterozygous state in any of the healthy relatives sequenced (Fig. 1A, 1B). The D171N (kindred A), A267V (kindreds B, D) D168A (kindred Q), N397D (kindred X), L351F (kindred AA) and T385M (kindred AB) mutations were found to have occurred *de novo* (a total of 7 patients from 7 kindreds), consistent with the complete clinical penetrance of all known CMCD-associated *STAT1* alleles. The mutations were not found in the NCBI, Ensembl and dbSNPs databases, or in our own in-house database of 700 exomes. They were also absent from the 1,052 controls from 52 ethnic groups in the *Centre d'Etude du Polymorphisme Humain* (CEPH) and Human Genome Diversity (HGD) panels (Cann *et al*., 2002; Jakobsson *et al*., 2008), suggesting that they were rare CMCD-causing variants rather than irrelevant polymorphisms.

The 17 new mutations affecting the CCD and DBD and the T385M and E353K mutations already described by Takezaki *et al.* and Romberg *et al*, were non conservative and were therefore predicted to affect protein structure and function. Moreover, most of the affected residues have been conserved throughout evolution, in the species in which *STAT1* has been sequenced. In addition, a new predictive tool, "Condel", which combines predictions from SIFT, Polyphen 2 and Mutation Assessor (Adzhubei *et al*., 2010; Chen *et al*., 2010; Gonzalez-Perez & Lopez-Bigas, 2011; Kumar *et al.,* 2009; Reva *et al*., 2011), identified 14 mutations as damaging, one as possibly damaging (D168E) and only three as neutral (Y287H, P329L, N357D) (Table S1). These genetic data, combined with the previous identification in other patients with CMCD of 13 mutations affecting the

CCD (Liu *et al*., 2011; Romberg *et al*., 2013) and two mutation affecting the DBD of STAT1 (Takezaki *et al.*, 2012; Romberg *et al.*, 2013), demonstrated experimentally to be GOF, and of one CCD mutation clinically associated with CMCD (I156T) (Romberg *et al*., 2013), suggest that the seven new mutations affecting the CCD and the 10 new mutations affecting the DBD may cause AD CMCD in these patients. However, the existence of loss-of-function (LOF) mutations underlying Mendelian susceptibility to mycobacterial disease (MSMD) in the close vicinity of the DBD mutations (Fig. 1A, 1C) (Averbuch *et al*., 2011; Chapgier *et al*., 2006) raises questions about the likelihood of these mutations being responsible for CMCD (i.e. whether they were GOF and, if so, by what mechanism).

We therefore carried out functional characterization for two CMCD-associated *STAT1* alleles with mutations affecting the DBD (R321G and N355D) (Fig. 1; Table 1). We compared these alleles not only with a wild-type (WT) and a previously characterized GOF CCD mutant allele (R274Q), but also with the two LOF DBD alleles (E320Q and Q463H) (Fig. 1A, 1C) (Boisson-Dupuis *et al*., 2012; Chapgier *et al*., 2006). We transfected STAT1-deficient U3C fibrosarcoma cells with the various *STAT1* alleles. On average, the responses to stimulation with IFN-γ, IFN-α or IL-27 in cells transfected with the CMCD-associated DBD and CCD alleles were three times stronger than those observed with the WT allele, as shown by measurement of the induction of gamma-activated sequence (GAS)-dependent reporter gene transcription activity, with mock-, E320Q- and Q463H-transfected cells serving as negative controls (Fig. 2A, Fig. S1A, S1B). Western blotting (WB) showed STAT1 tyrosine 701 phosphorylation levels to be higher for the R321G and N355D alleles than for the WT allele after stimulation with IL-

27, IFN-γ, or IFN-α, whereas total STAT1 protein levels were similar in all cells tested, as were STAT3 protein and phosphorylation levels (Fig. 2B; Fig. S1C). Accordingly, we observed an increase in GAS-binding activity upon stimulation with IL-27, IFN-γ or IFN-α in cells transfected with the R321G or N355D allele, as assessed by electrophoretic mobility shift assay (EMSA) (Fig. 2B, Fig. S1D). The transcription of the *CXCL9* and *CXCL10* target genes was also greatly enhanced by IFN-γ stimulation (Fig. 2C, 2D). Finally, higher levels of STAT1 phosphorylation and GAS-binding activity were also observed when U3C cells were transfected with six additional CMCD-associated STAT1 DBD alleles (C324R, L351F, E353K, K388E, M390T, N397D) (Fig. S2E). Neither U3C cells transfected with the WT allele nor those transfected with any of the *STAT1* mutant alleles responded to stimulation with high concentrations of IFN-λ in similar conditions (data not shown).

We then compared baseline and IFN-dependent transcriptional activity in transfected U3C cells, for the 31 CMCD-associated *STAT1* alleles. We used this *in vitro* system to assess the IFN-γ-dependent GAS transcriptional activity of all 31 the CMCD-associated *STAT1* alleles All CMCD-associated alleles were GOF in terms of GAF transcriptional activity, including the three predicted to be neutral by Condel (Y287H, P329L, N357D). Levels of activity were, on average, three times those with the WT allele, with the E320Q and Q463H alleles used as negative controls (Fig. S2A, S2B). The mutant alleles differed in their activity levels, which ranged from two to four times those for the WT allele. Moreover, the ranking of the mutations depended on the amount of IFN-γ used (10 or 1,000 IU/ml). We then assessed ISRE transcriptional activity in response to stimulation with various doses of IFN-α under the same conditions. We

initially reported no clear GOF for the three CCD alleles tested in a previous study (Liu *et al*., 2011), but we detected a mean two-fold increase in activity in cells transfected with CMCD-associated *STAT1* alleles under optimized conditions (See Materials and Methods) (Fig. S2C, S2D). In this assay, all but one of the CMCD-associated mutations (K388E being the exception), were GOF in terms of ISRE transcriptional activity. An increase in ISRE activity in response to IFN-α was observed in cells transfected with CMCD-associated alleles, using various reporter plasmids containing the ISRE sequence (data not shown). Thus, our results demonstrate that the 31 CMCD-associated *STAT1* alleles described in this manuscript are intrinsically GOF, at least for IFN-γ-dependent transcriptional activity, but also for IFN-α/β-dependent activity in all but one case.

The DBD of STAT1 is known to be involved not only in the DNA-binding activity of STAT1, but also in the nuclear-cytoplasmic transport and nuclear dephosphorylation of STAT1 (Begitt *et al*., 2000; Chen *et al*., 1998; Fagerlund *et al*., 2002; Haspel & Darnell, 1999; McBride *et al*., 2002; McBride *et al*., 2000; Melen *et al*., 2001; Meyer *et al*., 2002). The CMCD-associated *STAT1* CCD alleles have been shown to be GOF due to the impairment of STAT1 dephoshorylation in the nucleus (Liu *et al*., 2011; Takezaki *et al.,* 2012). We also documented an increase in Tyr 701 phosphorylation in response to IL-27, IFN-α and IFN-γ, for CMCD-associated DBD STAT1 alleles, on western blots (Fig. 2B, Fig. S1C). We observed no constitutive STAT1 Tyr701 phosphorylation in cells transfected with the CMCD-associated DBD or CCD alleles, in which STAT3 was normally activated (Fig. 2B, Fig. S1C, data not shown). The mechanism underlying the gain of phosphorylation was explored in cells transfected with the N355D allele and treated with the tyrosine kinase inhibitor staurosporine or the

phosphatase inhibitor pervanadate. The dephosphorylation of IFN-γ- or IL-27-activated N355D STAT1 was impaired by staurosporine, to an extent similar to that for the known experimental dephosphorylation mutant Q340A (Mertens *et al*., 2006) (Fig. 2E, Fig. S2F). By contrast, pervanadate normalized the phosphorylation of N355D and other CMCD-associated STAT1 DBD-mutated proteins after stimulation with IFN-γ to WT levels (Fig. 2F, Fig. S2G). A known CMCD-causing CCD-mutated *STAT1* allele, R274Q, was used as a control (Fig. 2F). Thus, the CMCD-associated *STAT1* alleles with missense mutations affecting the DBD, like the CMCD-associated *STAT1* CCD missense alleles, are GOF, due to the impairment of dephosphorylation.

We investigated the association of the heterozygous DBD *STAT1* alleles with an AD cellular phenotype, by deriving EBV-transformed B cells (EBV-B cells) from a CMCD patient heterozygous for the N355D allele. IFN-γ-, IFN-α/β- and IL-27-dependent STAT1 phosphorylation in EBV-B cells from this patient was stronger than that in EBV-B cells from a healthy control (WT/WT), two MSMD patients carrying heterozygous *STAT1* LOF mutations affecting the DBD (Q463H/WT and E320Q/WT) and a patient with complete STAT1 deficiency ($STAT1^{-/-}$), as shown by western blotting (Fig. 3A, 3B). Despite the higher levels of STAT1 phosphorylation in the cytoplasm, phosphorylation levels were consistently higher in the nucleus of the N355D/WT EBV-B cells than in the WT/WT control cells, following stimulation with IFN-γ or IFN-α (Fig. S3A). Moreover, we observed stronger GAF DNA-binding activity in response to stimulation with IFN-γ-, IFN-α/β- or IL-27 in N355D/WT EBV-B cells than in WT/WT and Q463H/WT and E320Q/WT EBV-B cells, as shown by EMSA (Fig. 3C, 3D). The response to IFN-λ was assessed by measuring the transcription of the target gene *IFIT1*

(induction of interferon-induced protein with tetratricopeptide repeats 1), as previously described (Chapgier *et al*., 2009). EBV-B cells from CMCD patients with *STAT1* mutations displayed a high degree of variability in terms of *IFIT1* mRNA induction, making it impossible to draw firm conclusions regarding the impact of the CMCD-associated *STAT1* alleles. Finally, despite the detection of a modest increase in ISRE transcriptional activity in U3C cells transfected with the CMCD-associated *STAT1* alleles (Fig. S2C, S2D), the IFN-α/β-dependent DNA-binding activity of ISGF3 in EBV-B cells from the patient tested (N355D/WT) appeared to be normal (Fig. S3B). Nevertheless, these data suggest that the heterozygous N355D/WT GOF allele is associated with an AD phenotype of enhanced STAT1- and GAF-dependent cellular responses to key STAT1-activating cytokines, such as IFN-α/β, IFN-γ and IL-27.

We then investigated cytokines that predominantly activate STAT3, rather than STAT1, such as IL-6, IL-21 and IL-23 (Bettelli *et al*., 2006; Kishimoto, 2005; Spolski & Leonard, 2008). As previously observed in the cells of a CMCD patient heterozygous for a CCD mutation (R274Q/WT) (Liu *et al.,* 2011), the levels of STAT1 phosphorylation in response to IL-6 and IL-21 were higher in the EBV-B cells of the patient heterozygous for the N355D allele than in those from a healthy control or from the two patients with MSMD due to mutations affecting the DBD, whereas STAT3 activation was normal, as shown by western blotting (Fig. 3E, 3F). Consistently, GAS activity in response to IL-6 and IL-21 was stronger in cells from the N355D/WT CMCD patient (Fig. 3G, 3H), than in WT/WT cells or cells from negative controls (E320Q/WT, Q463H/WT, $STAT1^{-/-}$), including cells heterozygous for LOF and dominant-negative mutations of *STAT3* ($STAT3^{+/-}$). These data suggest that heterozygous missense mutations affecting the DBD

of STAT1 are also GOF and confer an AD cellular phenotype of enhanced STAT1- and GAF-dependent cellular responses to cytokines predominantly activating STAT3, such as IL-6 and IL-21. We then investigated the response of the EBV-B cells to IL-23. No STAT1 phosphorylation was detected in controls or in CMCD patients and STAT3 phosphorylation varied considerably between the various positive controls tested, making it impossible to draw any firm conclusions (Fig. S3C, data not shown).

Having established that the newly discovered *STAT1* alleles were intrinsically GOF and conferred an AD cellular phenotype of hyper-responsiveness to IFNs and IL-27, we investigated the possible impairment of IL-17 T cell development in heterozygous patients. Indeed, we previously showed that patients with CMCD-causing *STAT1* CCD-mutated alleles displayed an impairment of IL-17-producing T-cell development from bulk leukocytes, as shown by *in vitro* and *ex vivo* studies comparing the cells of these patients with those from healthy controls or patients bearing mono- or bi-allelic LOF *STAT1* alleles. We thus determined the proportion of IL-17A- and IL-22-producing T cells by flow cytometry, and assessed the production of these cytokines by ELISA, in new patients with heterozygous missense CDD and DBD CMCD-associated *STAT1* alleles. Nine CMCD patients described in a previous study (Liu *et al*., 2011) were tested again (Fig. 4). The seven CMCD patients with *STAT1* DBD mutations and the 25 CMCD patients with *STAT1* CCD mutations tested had significantly ($p < 10^{-4}$) lower proportions of circulating IL-17A- and IL-22-producing T cells, as measured *ex vivo,* than 91 healthy controls tested in the same conditions (Figures 4A, 4B). Moreover, significantly ($p < 10^{-4}$) smaller amounts of IL-17A and IL-22 were secreted by freshly prepared leukocytes stimulated with PMA and ionomycin *ex vivo*, as shown by ELISA (Figures 4C and 4D).

Similarly, as previously shown for CCD mutations, PBMCs from CMCD patients carrying GOF *STAT1* DBD mutations (and GOF *STAT1* CCD mutations) cultured *in vitro* for five days in the presence of IL-6, IL-1β, TGF-β and IL-23, presented significantly lower proportions of IL-17A– and IL-22–producing T cells than PBMCs from controls ($p < 10^{-4}$) (Fig. S4A, S4B). Thus, CMCD patients heterozygous for GOF STAT1 DBD alleles, including the GOF N355D mutant allele, displayed lower levels of IL-17 and IL-22 production by peripheral T cells, providing a common immunological basis of disease, regardless of the location of the GOF *STAT1* mutations.

We then investigated whether the *STAT1* mutations affecting the CCD and DBD impaired the development of IL-17 T cells from naïve $CD4^+$ T cells *in vitro*. A combination of TGF-β and IL-6 has been shown to be essential for the initial differentiation of IL-17 T cells in mice (Bettelli *et al*., 2006; Kimura & Kishimoto, 2010; Mangan *et al*., 2006; Melton *et al*., 2010; Zhou *et al*., 2007), but the key cytokines required in humans remain less clearly defined (Sallusto *et al*., 2012). Various combinations of cytokines have been used for the differentiation of human IL-17 T cells: (1) TGF-β and IL-21; (2) IL-1β and IL-6; (3) IL-1β and IL-23 and (4) TGF-β and IL-23 (Acosta-Rodriguez *et al*., 2007; Cosmi *et al*., 2008; Korn *et al*., 2007; Santarlasci *et al*., 2009; Valmori *et al*., 2010; Yang *et al*., 2008). We thus purified $CD45RA^+$ $CD4^+$ T cells and cultured them in plates coated with an antibody (Ab) against CD3 and containing a soluble Ab against CD28, together with individual cytokines or all possible combinations of TGF-β, IL-1β, IL-6, IL-21 and IL-23, in the presence of IL-2. The proportion of IL-17A-expressing T cells and the secretion of IL-17A were measured from day 5 to 12 by flow cytometry and ELISA, respectively. We obtained the most reproducible results

within and between controls with a combination of IL-1β and IL-23 for 12 days (data not shown). IL-6 was not retained, as it increased interindividual variability. In these conditions, patients heterozygous for CCD or DBD *STAT1* mutations had lower ($p < 10^{-3}$) proportions of IL-17A T cells and secreted smaller amounts of IL-17A ($p < 10^{-2}$) (Fig. 5A, 5B). Impaired IL-17 T-cell development in these patients was similar to that seen in patients with other conditions conferring CMC, including AD-HIES patients with heterozygous LOF *STAT3* mutations and AR MSMD patients with biallelic LOF mutations of *IL12RB1* (Fig. S4C). Collectively, these data therefore demonstrate that the GOF *STAT1* mutations caused CMCD by impairing IL-17 T-cell immunity.

Finally, we investigated the mechanisms by which CMCD-causing GOF *STAT1* alleles prevent the development of IL-17 T cells. In the culture conditions defined above, we added a combination of IFN-α2a, IFN-β1a, IFN-γ and IL-27, which inhibits the development of IL-17 T cells via STAT1 in mice (Batten *et al*., 2006; Diveu *et al.,* 2009; El-behi *et al*., 2009; Feng *et al.,* 2008; Hirahara *et al*., 2012; Stumhofer *et al*., 2006; Villarino *et al.,* 2010; Yoshimura *et al.*, 2006) and humans (Amadi-Obi *et al*., 2007; Chen *et al*., 2009; Crabe *et al*., 2009; Guzzo *et al*., 2010; Liu & Rohowsky-Kochan, 2011; Ramgolam *et al*., 2009). We observed a large decrease in the proportion of IL-17A T cells and in the secretion of IL-17A, in both the healthy controls and the *STAT1* patients tested (Fig. 5C, 5D, left panel). The effect was statistically significant when measured both by flow cytometry and ELISA, for CMCD patients ($p < 10^{-3}$), and for controls ($p < 5 \times 10^{-3}$ and $p < 10^{-2}$ respectively). Moreover, in these inhibitory conditions, the difference in terms of IL-17A T cell proportion and IL-17A production between controls and patients was much more significant than in the absence of IFNs and IL-27 ($p < 10^{-3}$) (Fig.

5C, 5D right panels). At higher concentrations of IFN and IL-27, stronger inhibition was observed in the cells of controls and patients, with similar levels of inhibition in both (Fig. S4D). These data suggested that the poor development of IL-17 T cells in patients heterozygous for *STAT1* alleles might result, at least in part, from enhanced IFN-α/β, IFN-γ and IL-27 responses via STAT1. We tested this hypothesis, by treating the cells with a combination of neutralizing Abs against IFN-α/βR2, IFN-γ and IL-27. These Abs rescued the development of IL-17 T cells carrying GOF *STAT1* mutations, whereas this effect was not detectable in healthy cells (Fig. 5E, 5F, right panel). Indeed, the effect of these Abs reached significance only in the patients' cells ($p < 10^{-3}$ by flow cytometry and $p < 5. \times 10^{-4}$ by ELISA). Moreover, in these conditions, the difference between the cells of the controls and those of the patients was abolished (Fig. 5E, 5F, left panel). In these conditions, the proportion of $CD4^+$ IFN-γ^+ was slightly lower in the patients' cells, whereas the amounts of IFN-γ and IL-27 secreted were similar for controls and patients (Fig. S5). Overall, these experiments established that the poor development of IL-17 T cells in CMCD patients carrying GOF mutations affecting the CCD or DBD of *STAT1* involves STAT1-dependent inhibition via IFN-α/β, IFN-γ and/or IL-27.

Discussion

Our data identify *STAT1* as the major CMCD-causing gene. We report 19 new patients carrying CCD mutations previously described in other unrelated patients, nine patients carrying previously unknown mutations affecting the CCD, and the identification of 12 mutations (including 10 previously unknown mutations) affecting the DBD in 30 patients, bringing the number of known patients suffering from CMCD due to *STAT1*

mutations to 139, only 18 months after this condition was first described (Hori *et al.,* 2012; Liu *et al*., 2011; Romberg *et al*., 2013; Smeekens *et al*., 2011; Takezaki *et al.,* 2012; Toth *et al*., 2012; van de Veerdonk *et al.,* 2011; Wang *et al*., 2012). These patients originate from 74 kindreds and at least 23 countries. All 33 mutations are rare missense mutations. Paradoxically, there are now about five times more patients carrying known disease-causing GOF *STAT1* mutations (139 patients) than LOF *STAT1* mutations (24 patients), despite the LOF mutations having been described 10 years earlier, in 2001 (Boisson-Dupuis *et al*., 2012; Chapgier *et al*., 2006; Chapgier *et al*., 2009; Dupuis *et al*., 2001; Hirata *et al*., in preparation; Kong *et al*., 2010; Kristensen *et al*., 2010; Sampaio *et al*., 2012; Tsumura *et al*., 2012; Vairo *et al*., 2011). Unlike the LOF mutations, up to 10 *STAT1* GOF mutations were recurrent (found in at least 2 families); M202V, M202I, A267V, R274W, R274Q, T288A, E353K, N357D, T385M, K388E (Hori *et al*., 2012; Liu *et al*., 2011; Romberg *et al*., 2013; Smeekens *et al*., 2011; Takezaki *et al.,* 2012; Toth *et al*., 2012; van de Veerdonk *et al*., 2011; Wang *et al*., 2012) and at least 10 occurred *de novo* (three of which were recurrent): D165H, D168E, Y170N, D171N, A267V, R274W, R274Q, N397D, L351F, T385M (Liu *et al*., 2011; Takezaki *et al*., 2012; Wang *et al*., 2012). There is therefore no founder effect, at odds with a previous suggestion (van de Veerdonk *et al*., 2011). Moreover, the clinical penetrance of these *STAT1* alleles is complete. However, the clinical presentation seems to vary between patients. For example, we have recently documented recurrent herpes virus disease in two relatives (Toth *et al*., 2012), reminiscent of AD-HIES patients with heterozygous LOF *STAT3* mutations (Chandesris *et al*., 2012; Siegel *et al*., 2011).

About 24 to 30% of patients (33 of 139) and GOF STAT1 kindreds (21 of 74) have mutations affecting the DBD. The discovery of 12 GOF mutations affecting the DBD of STAT1 was surprising, because all of the first 13 GOF mutations described affected the CCD (Liu *et al*., 2011; Romberg *et al*., 2013; Takezaki *et al*., 2012). In total, 20 GOF *STAT1* CCD alleles have now been identified. This discovery was also surprising because heterozygous LOF DBD mutations underlying mycobacterial disease had already been found in close proximity to these mutations (Averbuch *et al*., 2011; Boisson-Dupuis *et al*., 2012; Chapgier *et al*., 2006). However, we demonstrate here that the CMCD-causing mutations affecting the DBD behave like those affecting the CCD, as they are intrinsically GOF in terms of STAT1-dependent cellular responses to the cytokines activating predominantly STAT1 and those activating predominantly STAT3. They also confer an AD hyperresponsiveness to these cytokines in heterozygous cells. Finally, we demonstrated that patients with mutations affecting the DBD had impaired IL-17-producing T-cell development, as previously reported for mutations affecting the CCD. Given the key role played by IL-17 immunity in the development of CMCD (Cypowyj *et al*., 2012; Puel *et al*., 2011; Puel *et al*., 2012; Puel *et al*., 2010b), the low levels of IL-17 T cells generated probably accounts for the CMC in these patients. Overall, GOF alleles with mutations affecting the CCD and DBD seem to underlie biochemical, immunological and clinical phenocopies. The various alleles differ in their impact on STAT1 activity, but this impact does not seem to be correlated with the location of the mutations concerned in either of the domains. Perhaps more surprisingly, the variability in terms of biochemical STAT1 activity, at least for IFN-γ-dependent GAS-driven transcription, did not seem to be correlated with the IL-17 T-cell impairment documented

ex vivo and *in vitro* (Fig. S6). Biochemical or immunological variability may nevertheless underlie variability in clinical presentation, whether for infectious diseases or autoimmunity. An international clinical survey is underway to tackle this question.

Finally, we have unraveled a mechanism by which *STAT1* alleles with mutations affecting the DBD and CCD impair IL-17 T-cell development. We showed that naïve $CD4^+$ T cells from patients heterozygous for GOF *STAT1* mutations, like cells from patients heterozygous for LOF *STAT3* mutations, displayed impairment into IL-17 T cells *in vitro*. Cytokines such as IFN-α/β, IFN-γ, and IL-27 have been shown to inhibit the development of IL-17 T cells in a STAT1-dependent manner in mice (Batten *et al*., 2006; Diveu *et al.,* 2009; El-behi et al., 2009; Feng *et al*., 2008; Stumhofer *et al.,* 2006; Villarino *et al*., 2010; Yoshimura *et al*., 2006) and in healthy humans (Amadi-Obi *et al*., 2007; Crabe *et al*., 2009; Guzzo *et al*., 2010; Hirahara *et al.,* 2012; Liu & Rohowsky-Kochan, 2011; Ramgolam *et al*., 2009). The mechanism underlying this inhibition remains to be deciphered. We showed that the poor development of IL-17 T cells in STAT1-mutated CMCD patients results from higher than normal levels of STAT1-dependent stimulation by IFN-α/β, IFN-γ, and IL-27. Indeed, the IL-17 T cell phenotype was both aggravated by these cytokines and rescued by neutralizing Abs against them. The impact of both sets of experimental conditions was, indeed, much more pronounced in CMCD patients with *STAT1* mutations than in healthy controls. The GOF *STAT1* alleles therefore impair IL-17 T cell development by amplifying a physiological inhibitory mechanism. Other hypotheses, such as competition between STAT1 and STAT3 upon stimulation with the STAT3-dependent cytokines required for the development of IL-17 T cells, such as IL-6, IL-21 and IL-23, are also possible. In any

case, heterozygosity for GOF *STAT1* alleles with mutations affecting the DBD and CCD domains of the protein results in enhanced T cell responses to IFN-α/β, IFN-γ and IL-27, impairing the development of IL-17 T cells and precipitating CMC in these patients.

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Materials and Methods

Molecular genetics

Genomic DNA and total RNA were extracted from fresh blood cells and cell lines, respectively, as previously described (Chen *et al*., 2009). Genomic DNA was amplified and sequenced for all exons and the flanking intron regions of *STAT1* by PCR. Amplicons were sequenced with the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and analyzed with an ABI 3130 capillary sequencer (Applied Biosystems, Foster City, CA). PCR and sequencing primers are available on request. Quantitative PCR was performed on EBV-B cells for *IFIT1* and *GUS,* as previously described (Chapgier *et al*., 2006). U3C transfectants were stimulated by incubation with 10^3 IU/ml IFN-γ for 2 or 8 hours and subjected to RNA extraction. The cDNA was synthesized directly with random primers, by reverse transcription. *CXCL9* and *CXCL10* mRNA levels were assessed by quantitative PCR on cDNA, with TaqMan probes. The results were normalized with respect to the values obtained for the endogenous *GUS* cDNA.

Cell culture, plasmids and reagents

Epstein-Barr virus-immortalized B cells (EBV B cells) and the STAT1-deficient fibrosarcoma U3C cell line were cultured as previously described (Chapgier *et al*., 2009). IFN-γ (Imukin; Boehringer Ingelheim), IFN-α2b (IntronA; Schering-Plough), IL-27 (R&D Systems), IFN-λ (R&D Systems), IL-21 (R&D Systems), IL-23 (R&D Systems)

and IL-6 (R&D Systems) were used to stimulate cells at various concentrations and for various periods, as indicated.

The wild-type and the various mutant *STAT1* alleles, generated by site-directed mutagenesis (kit from Stratagene) with the *ad hoc* mismatched primers, were inserted into the pcDNA3-V5 expression vector (Chapgier *et al*., 2006; Kong *et al*., 2010). U3C cells were plated 12 hours before transfection, at a density of 2.5×10^5 cells/well in six-well plates, and transfected with 5 μg per plate of the various *STAT1* allele-encoding vectors, with the Calcium Phosphate Transfection Kit (Invitrogen).

Luciferase reporter assay

U3C cells were dispensed into 96-well plates (1×10^4/well) and transfected with reporter plasmids (CignalTM GAS and ISRE Reporter Assay Kit, SABiosciences, Maryland, USA; PathDetect ISRE cis Reporting System, Catalog #219092, Agilent Technologies) and 100 ng/well (instead of the 20 ng/well previously used for transfection to test ISRE activity; Chapgier *et al*., 2006)) of plasmids carrying the various *STAT1* alleles or an empty vector, in the presence of Lipofectamine LTX (Invitrogen). Six hours after transfection, we replaced the medium with DMEM containing 10% FBS. Twenty-four hours after transfection, the transfectants were stimulated by incubation with IFN-γ (10 IU/ml or 1,000 IU/ml), IL-27 (100 ng/ml), IFN-λ1 (100 ng/ml) and IFN-α2b (5,000 IU/ml) for 8 hours and subjected to luciferase assays with the Dual-Glo luciferase assay system (Promega). Experiments were performed in triplicate and firefly luciferase activity was normalized with respect to *Renilla* luciferase activity. The data are expressed as fold induction with respect to non stimulated cells or in relative luciferase units (RLU).

Immunoblot analysis and electrophoretic mobility shift assays

The following optimal stimulation conditions were used. U3C cells or EBV-B cells were stimulated by incubation for 20 min with 100 μg/ml IL-21, 30 min (15 min for U3C cells) with 10^3 or 10^5 IU/ml IFN-γ or IFN-α2b, 15 min with 50 ng/ml IL-6 or 30 min (15 min for U3C cells) with 50 or 100 ng/ml IL-27. Western blotting was carried out as previously described (Liu *et al*., 2011). Briefly, cell activation was blocked with cold 1 x PBS, the cells were lysed in 1% NP-40 lysis buffer and the proteins were recovered and subjected to SDS-PAGE. We used antibodies directed against phosphorylated STAT1 (pY701; BD), STAT1 (C-24; Santa Cruz), α-tubulin (Santa Cruz), phosphorylated STAT3 (Cell Signaling), lamin B1 (Santa Cruz), GAPDH (Santa Cruz) and STAT3 (Santa Cruz). EMSA was carried out as previously described (Dupuis *et al*., 2003). Briefly, cell activation was blocked by incubation with cold 1 x PBS and the cells were gently lysed to remove cytoplasmic proteins without disrupting the nucleus. We then added nuclear lysis buffer and recovered the nuclear proteins, which were subjected to non denaturing electrophoresis with radiolabeled GAS (from the FCγR1 promoter: 5'-atgtatttcccagaaa-3') and ISRE (from the ISG15 promoter: 5'-gatcgggaaagggaaaccgaaactgaa-3') probes. U3C transfectants or EBV-B cells were stimulated with 10^5 IU/ml IFN-γ, 10^5 IU/ml IFN-α2b or 100 ng/ml IL-27 for 20 min (15 min for U3C cells) and the nuclear proteins were then extracted, as described above.

Staurosporine and pervanadate treatment of cells

We assessed dephosphorylation by stimulating U3C transfectants with 10^5 IU/ml IFN-γ. The cells were then washed and incubated with 1 μM staurosporine in DMEM for 15, 30 or 60 min. The cells were then lysed with 1% NP-40 lysis buffer, and the proteins recovered were subjected to immunoblot analysis. Pervanadate was prepared by mixing orthovanadate with H_2O_2 and incubating for 15 min at 22°C. U3C transfectants were treated with pervanadate (0.8 mM orthovanadate and 0.2 mM H_2O_2) five minutes before stimulation. They were then stimulated with IFN-γ for 15 min. The stimulation was stopped by adding cold 1 x PBS. The proteins were recovered and subjected to immunoblot analysis.

Extraction of nuclear and cytoplasmic proteins

U3C transfectants and EBV-B cells were stimulated with IFN-γ for 15 min and 20 min, respectively, and subjected to nuclear and cytoplasmic protein extraction with NE-PER Nuclear and Cytoplasmic Extraction Regents (Thermo Scientific), according to the manufacturer's protocol.

Modeling

Images of the three-dimensional structure were generated from the coordinates of the homodimer STAT1 (PDB# 1BF5) (Chapgier *et al*., 2006) and displayed with PDB viewer (http://www.expasy.org/spdbv/) (Chen *et al*., 1998), using the PDB code 1BF5.

Evaluation of IL-17A- and IL-22-producing T cells

IL-17A- and IL-22-producing T cells were evaluated by intracellular staining or by ELISA, as previously described (Guex & Peitsch, 1997). Briefly, PBMCs were purified by centrifugation on a gradient (Ficoll-Paque PLUS; GE Healthcare) and resuspended in RPMI supplemented with 10% FBS (RPMI/10% FBS; Invitrogen). Adherent monocytes were removed from the PBMC preparation by incubation for 2 h at 37°C, under an atmosphere containing 5% CO_2.

Analysis of IL-17-producing T cells

For the *ex vivo* evaluation of IL-17- and IL-22-producing T cells, 5×10^6 nonadherent cells were resuspended in 5 ml RPMI/10% FBS in 25 cm^2 flasks and stimulated by incubation with 40 ng/ml PMA (Sigma-Aldrich) and 10^{-5} M ionomycin (Sigma-Aldrich) in the presence of a secretion inhibitor (1 µl/ml Golgiplug; BD Biosciences), for 12 hours. Flow cytometry analysis was performed as described below.

For *in vitro* evaluation of the IL-17– and IL-22–producing T cell blasts after differentiation, the nonadherent PBMCs were dispensed into 24-well plates at a density of 2.5×10^6 cells/ml in RPMI/10% FBS and activated with 2 µg/ml of an antibody directed against CD3 (Orthoclone OKT3; Janssen-Cilag) together with 5 ng/ml TGF-β1 (240-B; R&D Systems), 20 ng/ml IL-23 (1290-IL; R&D Systems), 50 ng/ml IL-6 (206-IL; R&D Systems), and 10 ng/ml IL-1β (201-LB; R&D Systems). After three days, the cells were transferred to a new plate and restimulated in the same activation conditions, except that the anti-CD3 antibody was replaced with 40 IU/ml IL-2 (Proleukin i.v.; Chiron). We added 1 ml of the appropriate medium, resuspended the cells by gentle pipetting, and then split the cell suspension from each well in two. Flow cytometry was

performed on one of the duplicated wells two days later, after stimulation by incubation for 12 hours with 40 ng/ml PMA and 10^{-5} M ionomycin in the presence of 1 µl/ml GolgiPlug. Flow cytometry analysis was performed as described below. The contents of the other duplicate well were split in two, with one half left unstimulated and the other stimulated by incubation with 40 ng/ml PMA and 10^{-5} M ionomycin for another two days. Supernatants were collected after 48 h of incubation for ELISA.

For the *in vitro* differentiation of IL-17-producing T cells from $CD45RA^+/CD4^+$ T cells in the presence or absence of a cocktail of type I and type II IFNs and IL-27 or a cocktail of neutralizing antibodies against IFNs and IL-27, $CD45RA^+$ $CD4^+$ T cells were isolated from nonadherent PBMCs by negative selection on MicroBeads (naïve $CD4^+$ T-cell isolation kit II, 130-094-131, Miltenyi Biotec). The percentage of $CD45RA^+$ $CD4^+$ cells was over 95%. These cells were cultured in 48-well plates (1×10^6 cells/ml) coated with anti-CD3 antibody (1 µg/ml, Orthoclone OKT3; Janssen-Cilag), in the presence of soluble anti-CD28 antibody (1 µg/ml, 16-0289-85, eBioscience), IL-23 (20 ng/ml, 1290-IL; R&D Systems) and IL-1β (10 ng/ml, 201-LB; R&D Systems). After 2 days, cells were transferred to a new plate and restimulated with IL-2 (40 IU/ml, Proleukin i.v.; Chiron), together with IL-23 and IL-1β (20 ng/ml and 10 ng/ml, respectively), every 2 to 3 days for 10 days. In addition, cells were cultured under these conditions in the presence or absence of a cocktail of IFN-α2a (10 IU/ml, 11100-1, PBL InterferonSource), IFN-β1a (10 IU/ml, 11410-2, PBL InterferonSource), IFN-γ (10 IU/ml, Imukin, Boehringer Ingelheim) and IL-27 (1 ng/ml, 14-8279, eBioscience) or a cocktail of anti-IFN-α/βR2 (1 µg/ml, MAB1155, Chemicon), anti-IFN-γ (1 µg/ml, 16-7318-85, eBioscience) and anti-IL27 (1 µg/ml, AF2526, R&D Systems) antibodies or a cocktail of isotype controls of

these antibodies. With the exception of anti-CD3 and anti-CD28 antibodies, which were added only once, on day 0, all cytokines and antibodies were added to the culture medium every two or three days. After 12 days, cells were stimulated with by incubation with PMA (40 ng/ml, Sigma-Aldrich) and ionomycin (10^{-5} M, Sigma-Aldrich) for 48 h for ELISA, and for 12 h in the presence of GolgiPlug (1 µl/ml, BD) for flow cytometry analysis.

Flow cytometry

For flow cytometry analysis, cells were washed in cold PBS and surface-labeled by incubation with PECy5-conjugated anti-human CD3 antibody (BD Biosciences, 555334) in 2% FBS in PBS for 20 min on ice. Cells were then washed twice with 2% FBS in cold PBS, fixed by incubation with 100 µl of BD Cytofix for 30 min on ice and washed twice with BD Cytoperm (BD Cytofix/Cytoperm Plus, fixation/permeabilization kit, 555028, BD Biosciences). Cells were then incubated for 1 hour on ice with FITC-conjugated anti-human IL-17A (130-094-520, Miltenyi Biotec), PE-conjugated anti-human IL-22 (R&D Systems, IC7821P) or PE-conjugated anti-human IFN-γ (Myltenyi, 130-097-940) antibodies, washed twice with BD Cytoperm and analyzed with a BD FACSCanto II system.

ELISA

IL-17A, IL-22, IL-27 and IFN-γ levels were determined by ELISA, on the supernatants harvested after 48 hours of stimulation of whole-blood or *in vitro*-differentiated cells with 40 ng/ml PMA and 10^{-5} M ionomycin. We used anti-human IL-

17A, anti-human IL-22 and anti-human IL-27 Duoset kits (R&D Systems), and the anti-human IFN-γ kit (Sanquin, M9333), according to the manufacturer's instructions.

Statistical analysis

We assessed the differences between controls and CMCD patients bearing gain-of-function *STAT1* alleles under the various conditions, with the nonparametric Mann-Whitney test. Differences within CMCD or control groups between two experimental conditions (e.g. *in vitro* IL-17 T cell differentiation in the presence or absence of a cocktail of inhibitors, or *in vitro* IL-17 T cell differentiation in the presence or absence of a cocktail of neutralizing antibodies) were evaluated by paired tests: the Wilcoxon matched-pairs signed rank test. For all analyses, $p < 0.05$ was considered statistically significant.

Legends to figures

Figure 1. Heterozygous missense mutations affecting the STAT1 DNA binding domain in kindreds with AD CMCD. A. The human *STAT1* alpha isoform is shown, with its known pathogenic mutations. Coding exons are numbered with Roman numerals and delimited by a vertical bar. Regions corresponding to the coiled-coil domain (CCD), DNA-binding domain (DBD), linker domain (L), SH2 domain (SH2D), tail segment domain (TSD), and transactivator domain (TAD) are indicated, together with their amino-acid boundaries, and are delimited by bold lines. Tyr701 (pY) and Ser727 (pS) are indicated. Mutations in green are dominant and associated with partial STAT1 deficiency and Mendelian susceptibility to mycobacterial disease (MSMD). Mutations in brown are recessive and associated with complete STAT1 deficiency and intracellular bacterial and viral disease. Mutations in blue are recessive and associated with partial STAT1 deficiency and intracellular bacterial and/or viral disease. Mutations in red are dominant, located in the region encoding the CCD and associated with a gain of function of STAT1 and CMCD. Mutations in violet are dominant, located in the region encoding the DBD and associated with gain of function of STAT1 and CMCD. New mutations are shown in bold. * published in (Romberg *et al*. 2013) **B**. Pedigrees of 20 newly identified families with heterozygous gain-of-function *STAT1* mutations affecting the CCD and of 18 families with heterozygous gain-of-function *STAT1* mutations affecting the DBD. Each kindred is designated by a letter (A to AL), each generation is designated by a roman numeral (I-II-III-IV), and each individual is designated by an Arabic numeral (each individual studied is identified by a code of this type, organized from left to right). Black indicates CMCD patients. The probands are indicated by an arrow. When tested,

the genotype for *STAT1* is indicated below the individual concerned. **C**. Localization of the residues affected by the missense mutations on the 3D structure of phosphorylated STAT1 in complex with DNA (1BF5). Cartoon backbone representation and Van der Waals representation for the following amino acids highlighted: in red and purple, amino acids located in the CCD and DBD, respectively, and mutated in patients with CMCD; in blue and green, amino acids mutated in patients with MSMD alone or associated with viral diseases and in brown, the DNA molecule.

Figure 2. The mutant R321G and N355D *STAT1* alleles are gain-of-phosphorylation and gain-of-function for GAF (gamma activated factor)-dependent cellular responses. U3C cells were transfected with an empty vector (Mock), a wild-type (WT) allele, a CMCD-causing *STAT1* allele with a mutation affecting the CCD (R274Q), two CMCD-causing *STAT1* alleles with mutations affecting the DBD (N355D and R321G) and two MSMD-causing *STAT1* alleles with mutations affecting the DBD (E320Q and Q463H). The responses to IFN-γ, IFN-α and IL-27 (**A**), and to IL-27 (**B**) were then evaluated by determining the luciferase activity generated from a reporter gene under the control of the GAS promoter (**A**), and by assessing STAT1 and STAT3 phosphorylation by western blotting (WB) and DNA-binding activity by electrophoretic mobility shift assays (EMSA) with a GAS probe **(B)**. Experiments were performed at least three times, independently. **C and D**. Reverse-transcription-quantitative PCR was used to measure the induction of *CXCL9* (**C**) and *CXCL10* (**D**) 2 and 8 hours after stimulation with IFN-γ. Experiments were performed at least three times, independently. **E**. The dephosphorylation of STAT1 was assessed by WB in U3C cells transfected with a

mock vector, wild-type (WT) *STAT1*, the N355D or the Q340A (a known loss-of dephosphorylation mutant (Mertens et al, 2006)) *STAT1* mutant alleles were cultured in the presence of the tyrosine kinase inhibitor staurosporine for the indicated periods (0 to 60 min), after 15 min of IFN-γ stimulation. Three independent experiments were performed. **F.** WB of cytoplasmic and nuclear fractions of U3C cells transfected with a WT, R274Q or N355D allele of *STAT1,* and treated with IFN-γ with or without the phosphatase inhibitor pervanadate. Two independent experiments were performed.

Figure 3. The mutant N355D *STAT1* allele is dominant for GAF (gamma-interferon activated factor)-dependent cellular responses in EBV-B cells. EBV-B cells from a CMCD patient with a *STAT1* mutation (N355D/WT), healthy controls (WT/WT), two MSMD patients (STAT1 E320Q/WT and Q463H/WT), a patient with complete STAT1 deficiency ($STAT1^{-/-}$) and one AD-HIES patient ($STAT3^{+/-}$) were stimulated with IFN-γ (**A, C**), IFN-α (**A, C**), IL-27 (**B, D**), IL-6 (**E, F, G**) or IL-21 (**E, F, H**). pSTAT1 and pSTAT3 levels were determined independently at least twice, by western blotting WB (**A**, **B**, **E** and **F**) with an antibody recognizing either the Tyr701-phosphorylated STAT1 or the Tyr 705-phosphorylated STAT3. GAF DNA-binding activity upon stimulation was evaluated at least twice by EMSA with a GAS probe (**C, D, G, H**).

Figure 4. Impaired development and function of IL-17- and IL-22-producing T cells *ex vivo* in patients with AD CMCD and *STAT1* GOF mutations. Each symbol represents a value from a healthy control individual (black circles), a patient bearing a

STAT1 gain-of-function (GOF) mutation affecting the CCD (red circle), a patient bearing a *STAT1* GOF mutation affecting the DBD (violet circle). (**A, B**) Percentage of $CD3^+$ IL-$17A^+$ (**A**) and $CD3^+$ IL-22^+ (**B**) cells, as determined by flow cytometry, in nonadherent PBMCs activated by incubation for 12 hours with PMA and ionomycin. (**C, D**) Secretion of IL-17A (**C**) and IL-22 (**D**) by whole blood cells, as determined by ELISA, in the absence of stimulation (open symbols) and after stimulation with PMA and ionomycin for 48 h (closed symbols). Healthy controls are shown in black, CMCD patients with mutations affecting the STAT1 CCD are represented in red and patients with mutations affecting the STAT1 DBD are shown in violet. Horizontal bars represent medians. The *p* values for nonparametric Wilcoxon tests comparing patients with *STAT1* GOF mutations (*n* = 7 affecting the DBD, and *n*=25 affecting the CCD) and controls (*n* = 91) are indicated.

Figure 5. The differentiation of naïve $CD45RA^+$ $CD4^+$ T cells into IL-17-producing T cells *in vitro* is impaired in patients with AD CMCD and *STAT1* GOF mutations, due to enhanced STAT1 responses to IFNs and IL-27. (**A-B**) Proportion of IL-17A-producing T cells (**A**) and IL-17A secretion (**B**), after 12 days of naïve CD4 T cell differentiation in the presence of anti-CD3 and anti-CD28 antibodies, IL-1β and IL-23, as determined by flow cytometry and ELISA, respectively, after stimulation with PMA and ionomycin for 12 hours for flow cytometry analysis, and in the absence of stimulation (open symbols) or after 48 hours of stimulation (closed symbols) for ELISA analysis. Each symbol represents a value from a healthy control individual (black circles), a patient bearing a *STAT1* gain-of-function (GOF) mutation affecting the CCD (red

circles) or a patient bearing a *STAT1* GOF mutation affecting the DBD (violet circles). Horizontal bars represent medians. The *p*-values for the nonparametric Wilcoxon tests are shown for comparisons of patients with *STAT1* GOF mutations (*STAT1* CCD: *n* = 11 and *STAT1* DDB: *n* = 4) and controls (*n* = 37) (**A**) patients with *STAT1* GOF mutations (*STAT1* CCD: *n* = 9 and *STAT1* DDB: *n* = 3) and controls (*n* = 13) (**B**). (**C-D**) Development of IL-17-producing CD4 T cells (**C**) and IL-17A production (**D**) from naïve $CD45RA^+$ CD4 T cells, after 12 days of differentiation under IL-17 T cell-inducing conditions, in the presence of a cocktail of IFNs and IL-27 (IFN-α2a:10 IU/ml, IFN-β1a: 10 IU/ml, IFN-γ: 10 IU/ml and IL-27: 1 ng/ml), as determined by flow cytometry and ELISA. The left panel shows the impact of the cytokine cocktail on each individual, and the median values are shown on the right. Healthy controls are shown in black, CMCD patients with mutations affecting the STAT1 CCD are shown in red and patients with mutations affecting the STAT1 DBD are shown in violet. Horizontal bars represent the medians. The *p* values for nonparametric Wilcoxon tests comparing patients with *STAT1* GOF mutations (*n* = 3 affecting the DBD, and *n* = 12 affecting the CCD) and controls (*n* = 13) are indicated. (**E-F**) Development of IL-17-producing CD4 T cells (**E**) and IL-17A production (**F**) from naïve $CD45RA^+$ CD4 T cells, after 12 days of differentiation under IL-17 T cell-inducing conditions, in the presence of a cocktail of neutralizing antibodies (Abs) against IFNs and IL-27 (anti-IFN-α/βR2: 1 μg/ml, anti-IFN-γ: 1 μg/ml and anti-IL27: 1 μg/ml) or a cocktail of isotype control antibodies, as determined by flow cytometry and ELISA. The impact of the Abs cocktail on each individual is shown on left and the median values are shown on the right. Healthy controls are shown in black, CMCD patients with mutations affecting the STAT1 CCD are shown in red and patients

with mutations affecting the STAT1 DBD are shown in violet. Horizontal bars represent medians. The *p* values for nonparametric Wilcoxon tests comparing patients with *STAT1* GOF mutations (*n* = 3 affecting the DBD, and *n* = 13 affecting the CCD) and controls (*n* = 11) are indicated. Differences within CMCD or control groups between two experimental conditions (e.g. *in vitro* IL-17 T cell differentiation in the presence or absence of a cocktail of inhibitors, or *in vitro* IL-17 T cell differentiation in the presence or absence of a cocktail of neutralizing antibodies) were evaluated by paired tests: the Wilcoxon matched-pairs signed rank test.

Table 1: Summary of the clinical and genetic data for the patients

Supplementary Figure 1: A-B. The response after 8 hours of stimulation with various doses of IL-27 (0.1, 0.2, 1, 2, 10, 20, 100 ng/ml) (**A**) or IFN-α (1, 5, 10, 50, 100, 500, 1000 IU/ml) (**B**) was evaluated by determining the activity of the luciferase reporter genes under the control of the GAS promoter in U3C cells transfected with a mock vector, a wild-type (WT) *STAT1* allele or the mutant *STAT1* alleles (R274Q, N355D or M390T). The response to IFN-γ (10^5 IU/ml for 15 min) and IFN-α (10^5 IU/ml for 15 min) **(C-D)** was evaluated by western blotting (**C**) and by EMSA (**D**) in U3C cells transfected with a mock vector, a wild-type (WT) *STAT1* form, the mutant CMCD-causing *STAT1* alleles (R321G, N355D, R274Q), or the MSMD-causing *STAT1* alleles (E320Q and Q463H). Experiments were performed at least three times, independently.

Supplementary Figure 2: A-B. The response to IFN-γ (10 or 1000 IU/ml for 16 hours) was evaluated by determining the luciferase activity (RLU, relative luciferase units) generated from a reporter gene under the control of the GAS promoter in U3C cells

transfected with a mock vector, a WT allele of *STAT1*, 29 (**A**) and 2 (**B**) different CMCD-causing mutant forms of *STAT1* and the MSMD-causing mutant alleles, E320Q and Q463H (**A**) and Y701C (**B**). Experiments were performed in triplicate and at least three times, independently. **C-D.** The response to various doses of IFN-γ (100 and 1000 IU/ml for 8 hours) was evaluated by determining the luciferase activity (RLU) generated from reporter genes under the control of the ISRE promoter in U3C cells transfected with a mock vector, a wild-type (WT) form or 29 (**C**) and 2 (**D**) CMCD-causing mutant forms of *STAT1* and the MSMD-causing mutant alleles, E320Q and Q463H (**C**) and Y701C (**D**). Experiments were performed at least three times, independently. **E.** GAF-DNA binding activity (top) and western blot (bottom) of U3C cells transfected with mock, WT, C324R, L351F, E353K, K388E, M390T, N397D DBD CMCD-causing *STAT1* alleles and the Q463H MSMD-causing *STAT1* allele, left unstimulated (NS) or stimulated with IFN-γ (10^5 IU/ml for 15 min); the results shown are representative of at least two independent experiments. PV, Pervanadate. **F**. The dephosphorylation of STAT1 was tested by western blotting in U3C cells transfected with a mock vector, the wild-type (WT) *STAT1* allele, the N355D CMCD-causing *STAT1* allele or the known loss-of-dephosphorylation Q340A *STAT1* mutant allele and cultured in the presence of the tyrosine kinase inhibitor staurosporine for the indicated time periods (0 to 60 min), after 15 min of IL-27 stimulation. Three independent experiments were performed. **G.** GAF-DNA binding activity (top) and western blot (bottom) of U3C cells transfected with a WT, C324R, L351F, E353K, K388E, M390T, N397D or L706S allele of *STAT1,* and treated with IFN-γ (10^5 IU/ml for 15 min), with or without the phosphatase inhibitor pervanadate (PV). Two independent experiments were performed.

Supplementary Figure 3: A. The cytoplasmic and nuclear fractions of EBV-B cells from a control (WT/WT), a CMCD patient (N355D/WT), one heterozygous patient with a dominant loss-of-function mutation of *STAT1* affecting the DBD (E320Q/WT), and a patient with complete STAT1 deficiency ($STAT1^{-/-}$) stimulated with IFN-γ and IFN-α were tested for the presence of phosphorylated STAT1 and total STAT1 by western blotting. **B.** The response of the patient's EBV-B cells (N355D/WT) was evaluated by EMSA with an ISRE probe and was compared with those of healthy controls (WT/WT), cells heterozygous for dominant loss-of-function mutations of *STAT1* (Q463H/WT and E320Q/WT) and cells with complete STAT1 deficiency ($STAT1^{-/-}$). Cells were stimulated with various doses of IFN-α; the results shown are representative of at least two independent experiments. **C.** EBV-B cells from a CMCD patient with a *STAT1* mutation (N355D/WT), two healthy controls (WT/WT1 and 2), two MSMD patients (STAT1 E320Q/WT and Q463H/WT), and one AD-HIES patient ($STAT3^{+/-}$) were stimulated with IL-23. STAT3 and pSTAT3 levels were determined independently, at least twice, by WB.

Supplementary Figure 4. Impaired development *in vitro* of IL-17-producing T cells from bulk PMBCs from healthy controls and CMCD patients with *STAT1* GOF mutations (A and B) Proportion of IL-17A producing $CD3^+$ T cells (**A**) or IL-22 producing $CD3^+$ T cells (**B**) after 5 days of differentiation of nonadherent PBMCs in the presence of IL-1β, IL-6, TGF-β and IL-23, as determined by flow cytometry. Each symbol represents a value from a healthy control individual (black circles), a CMCD patient bearing a *STAT1* GOF allele (affecting the CCD: red triangles, affecting the DBD: violet triangles). **(C) Impaired *in vitro* development of IL-17-producing T cells from**

naïve CD45RA$^+$ CD4$^+$ T cells in patients with AD CMCD due to STAT1 GOF, with AD HIES and STAT3 deficiency or with AR MSMD and IL-12Rβ1deficiency. Proportion of IL-17A-producing T cells after 12 days of naïve CD45RA$^+$ CD4 T cell differentiation in the presence of anti-CD3 and anti-CD28 antibodies, IL-1β, and IL-23, as determined by flow cytometry. Each symbol represents a value from an individual and horizontal bars represent medians. (**D**). **The differentiation of naïve CD45RA$^+$ CD4 T cells into IL-17-producing T cells *in vitro* is strongly inhibited by high doses of IFNs and IL-27 in cells from controls and from patients with a STAT1 GOF.** Proportion of IL-17A-producing T cells, after 12 days of naïve CD4 T-cell differentiation in the presence of anti-CD3 and anti-CD28 antibodies, IL-1β and IL-23, as determined by flow cytometry, after stimulation with PMA and ionomycin for 12 h, in the presence or absence of a cocktail of low doses of IFNs (10 IU/ml) and IL-27 (1 ng/ml) or a cocktail of high doses of IFNs (100 IU/ml) and IL-27 (10 ng/ml). The mean values were obtained for four healthy controls (left black columns) and four STAT1 GOF patients (right red columns).

Supplementary Figure 5. IFN-γ and IL-27 production after the differentiation of naïve CD45RA$^+$ CD4 T cells into IL-17-producing T cells *in vitro* is similar in patients and controls. Proportion of IFN-γ-producing T cells (**A**) and secretion of IFN-γ (**B**) and IL-27 (**C**), after 12 days of naïve CD4 T-cell differentiation in the presence of anti-CD3 and anti-CD28 antibodies, IL-1β and IL-23, as determined by flow cytometry and ELISA, after stimulation with PMA and ionomycin for 12 hours for flow cytometry analysis, and in the absence of stimulation (open symbols) or after 48 hours of stimulation (closed symbols) for ELISA analysis. Each symbol represents a

value from a healthy control individual (black circles), a patient bearing a *STAT1* gain-of-function (GOF) mutation affecting the CCD (red circles) or a patient bearing a *STAT1* GOF mutation affecting the DBD (blue circles). Horizontal bars represent medians. The *p*-values are shown for nonparametric Wilcoxon tests comparing patients with *STAT1* GOF mutations (*STAT1* CCD: $n = 11$ and *STAT1* DDB: $n = 4$) and controls ($n = 37$) (**A**) patients with *STAT1* GOF mutations (*STAT1* CCD: $n = 9$ and *STAT1* DDB: $n = 3$) and controls ($n = 13$) (**B**) patients with *STAT1* GOF mutations (*STAT1* CCD: $n = 9$ and *STAT1* DDB: $n = 3$) and controls ($n = 11$) (**C**).

Supplementary Figure 6. Absence of correlation between *STAT1* GOF mutations GAS-driven transcription and the impairment of IL-17 T–cell production *ex vivo* and *in vitro*. U3C cells were transfected with a wild-type (WT) or CMCD-causing *STAT1* allele. The responses to IFN-γ were evaluated by determining the luciferase activity generated by a reporter gene under the control of the GAS promoter (X axis), as a function of the proportion of IL-17A-producing T cells **(A)** or the amount of IL-17A produced **(B)** *ex vivo* in patients bearing the corresponding mutations (Y axis)**.** GAS activity is expressed as a function of the proportion of IL-17A-producing T cells **(C)** or the amount of IL-17A produced **(D)** in the *in vitro*-differentiated naïve $CD45RA^+$ CD4 T cells in patients bearing the corresponding mutations.

Supplementary Table 1: Predictions concerning the function of the mutant *STAT1* alleles associated with CMCD. Table summarizing, for the 19 mutations, the possible functional consequences (neutral, possibly deleterious or deleterious) as

predicted by Condel, which summarizes SIFT, Polyphen II and Mutation Assessor predictions.

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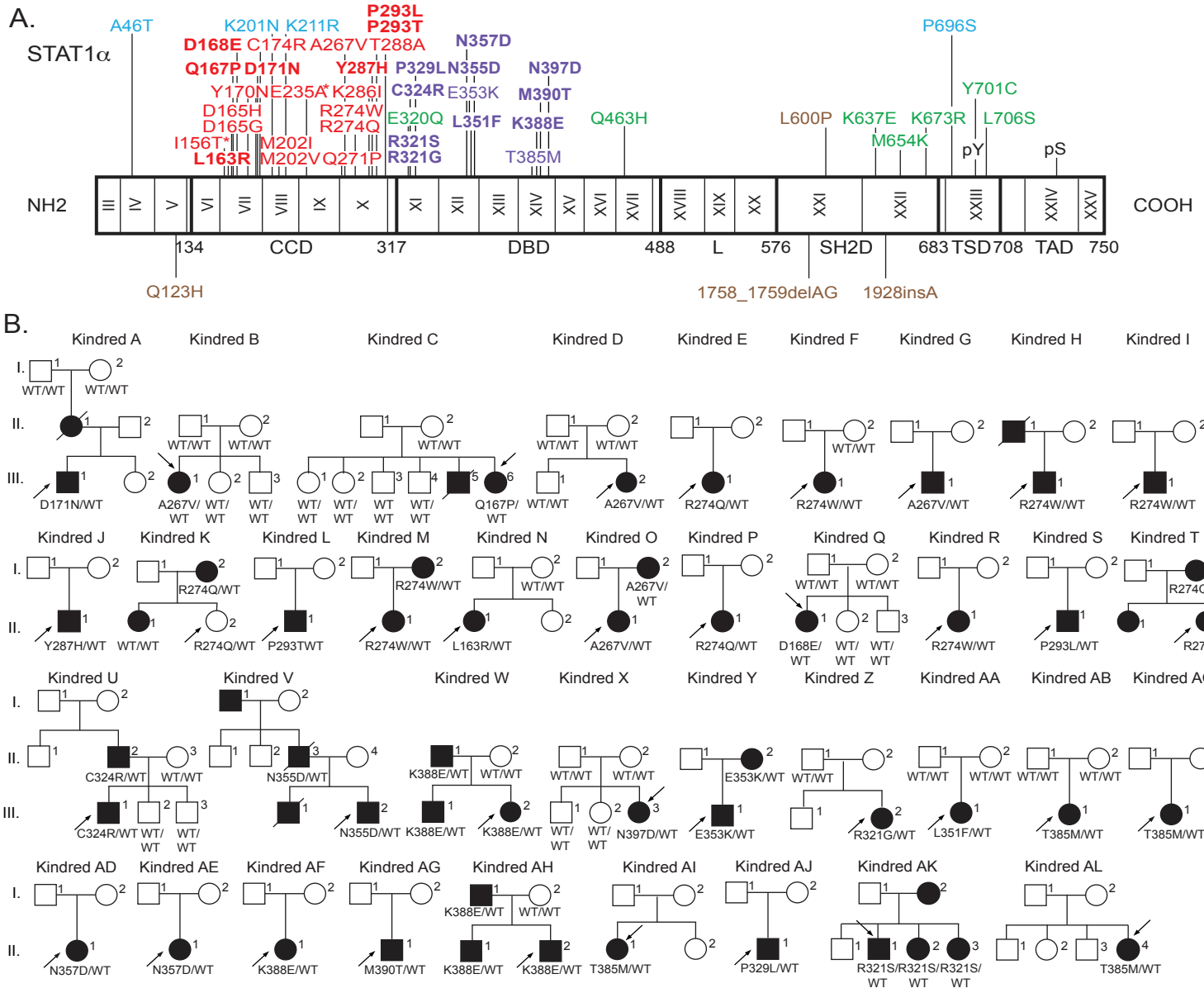
Figure 1


A.
STAT1α
A46T
K201N K211R
P293L
P293T
D168E C174R A267V T288A
N357D
Q167P D171N
Y287H
P329L N355D
N397D
Y170N E235A* K286I
C324R E353K
M390T
D165H
R274W
E320Q
L351F K388E
Q463H
D165G
R274Q
R321S
I156T* M202I
L163R M202V Q271P
R321G
T385M
L600P K637E K673R
P696S
Y701C
L706S
M654K
pY
pS
NH2
COOH
I II III IV V VI VII VIII IX X XI XII XIII XIV XV XVI XVII XVIII XIX XX XXI XXII XXIII XXIV XXV
134
CCD
317
DBD
488
L
576
SH2D
683
TSD
708
TAD
750
Q123H
1758_1759delAG
1928insA

B.

Kindred A
Kindred B
Kindred C
Kindred D
Kindred E
Kindred F
Kindred G
Kindred H
Kindred I
D171N/WT
A267V/ WT
Q167P/ WT
A267V/WT
R274Q/WT
R274W/WT
A267V/WT
R274W/WT
R274W/WT
Kindred J
Kindred K
Kindred L
Kindred M
Kindred N
Kindred O
Kindred P
Kindred Q
Kindred R
Kindred S
Kindred T
Y287H/WT
R274Q/WT
P293TWT
R274W/WT
L163R/WT
A267V/WT
R274Q/WT
D168E/ WT
R274W/WT
P293L/WT
R274Q/WT
Kindred U
Kindred V
Kindred W
Kindred X
Kindred Y
Kindred Z
Kindred AA
Kindred AB
Kindred AC
C324R/WT
N355D/WT
K388E/WT
N397D/WT
E353K/WT
R321G/WT
L351F/WT
T385M/WT
T385M/WT
Kindred AD
Kindred AE
Kindred AF
Kindred AG
Kindred AH
Kindred AI
Kindred AJ
Kindred AK
Kindred AL
N357D/WT
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T385M/WT
P329L/WT
T385M/WT

C.

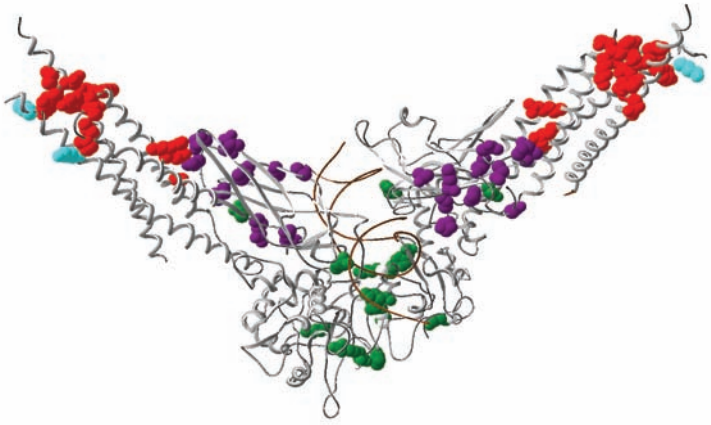


Figure 2

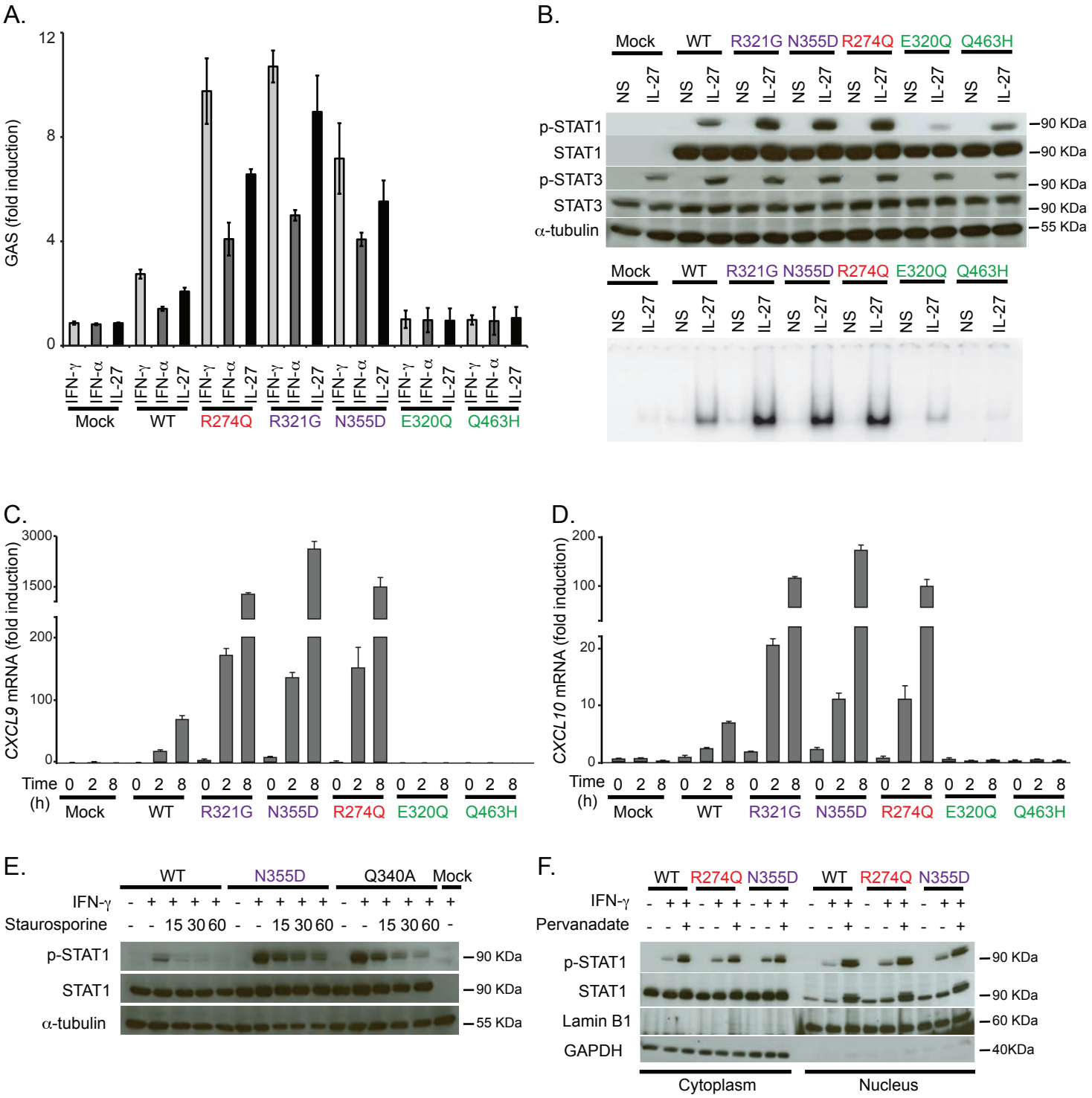

A.
GAS (fold induction)
12
8
4
0
IFN-γ
IFN-α
IL-27
Mock
WT
R274Q
R321G
N355D
E320Q
Q463H
B.
Mock
WT
R321G
N355D
R274Q
E320Q
Q463H
NS
IL-27
p-STAT1
STAT1
p-STAT3
STAT3
α-tubulin
90 KDa
90 KDa
90 KDa
90 KDa
55 KDa
C.
CXCL9 mRNA (fold induction)
3000
1500
200
100
0
Time (h)
0 2 8
Mock
WT
R321G
N355D
R274Q
E320Q
Q463H
D.
CXCL10 mRNA (fold induction)
200
100
20
10
0
E.
WT
N355D
Q340A
Mock
IFN-γ
Staurosporine
15 30 60
p-STAT1
STAT1
α-tubulin
90 KDa
90 KDa
55 KDa
F.
WT
R274Q
N355D
IFN-γ
Pervanadate
p-STAT1
STAT1
Lamin B1
GAPDH
90 KDa
90 KDa
60 KDa
40KDa
Cytoplasm
Nucleus

Figure 3

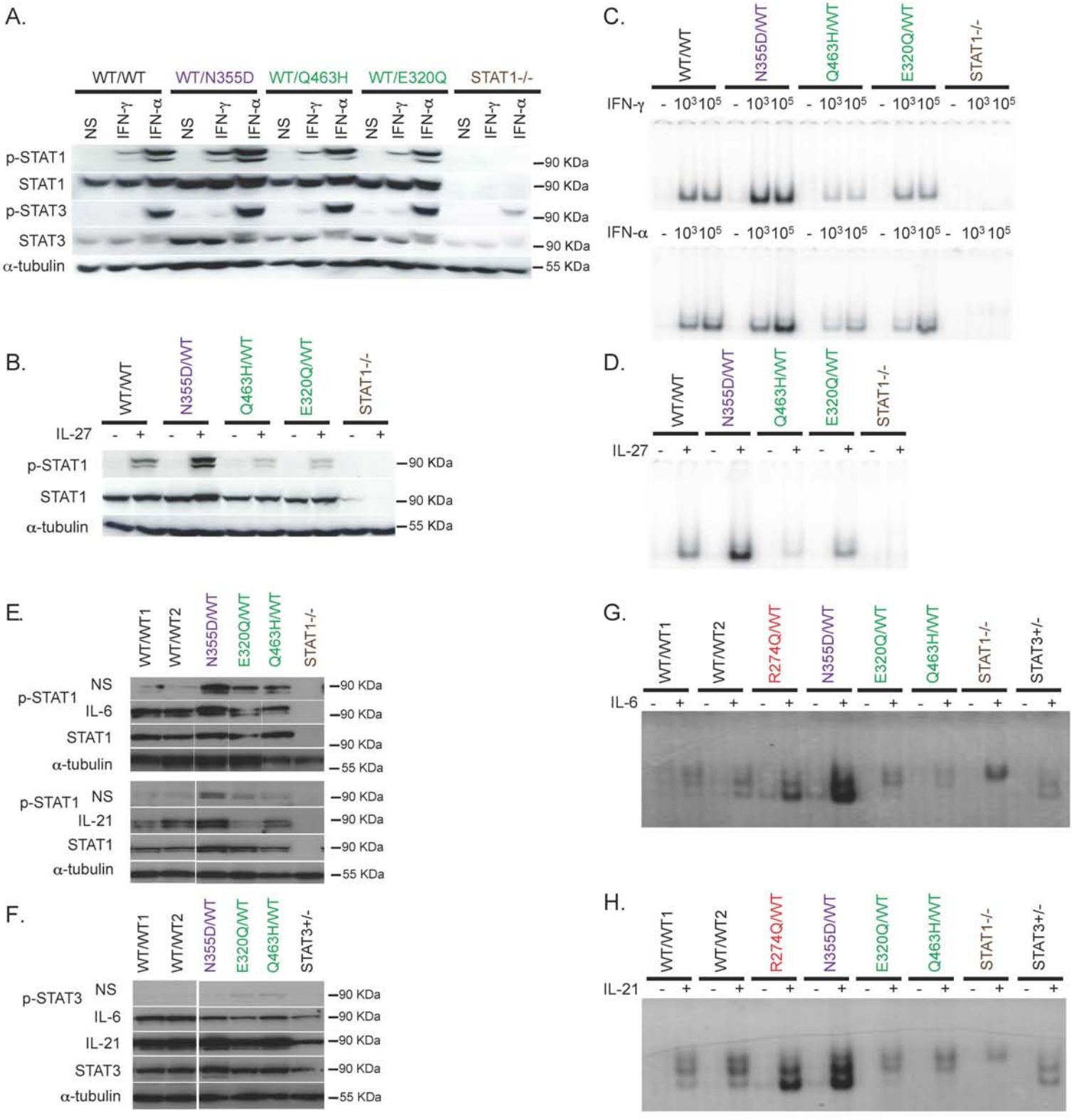

A.
WT/WT
WT/N355D
WT/Q463H
WT/E320Q
STAT1-/-
NS
IFN-γ
IFN-α
p-STAT1
STAT1
p-STAT3
STAT3
α-tubulin
90 KDa
55 KDa
B.
WT/WT
N355D/WT
Q463H/WT
E320Q/WT
STAT1-/-
IL-27
C.
IFN-γ
IFN-α
D.
E.
WT/WT1
WT/WT2
N355D/WT
E320Q/WT
Q463H/WT
STAT1-/-
IL-6
IL-21
F.
STAT3+/-
G.
R274Q/WT
H.

Figure 4

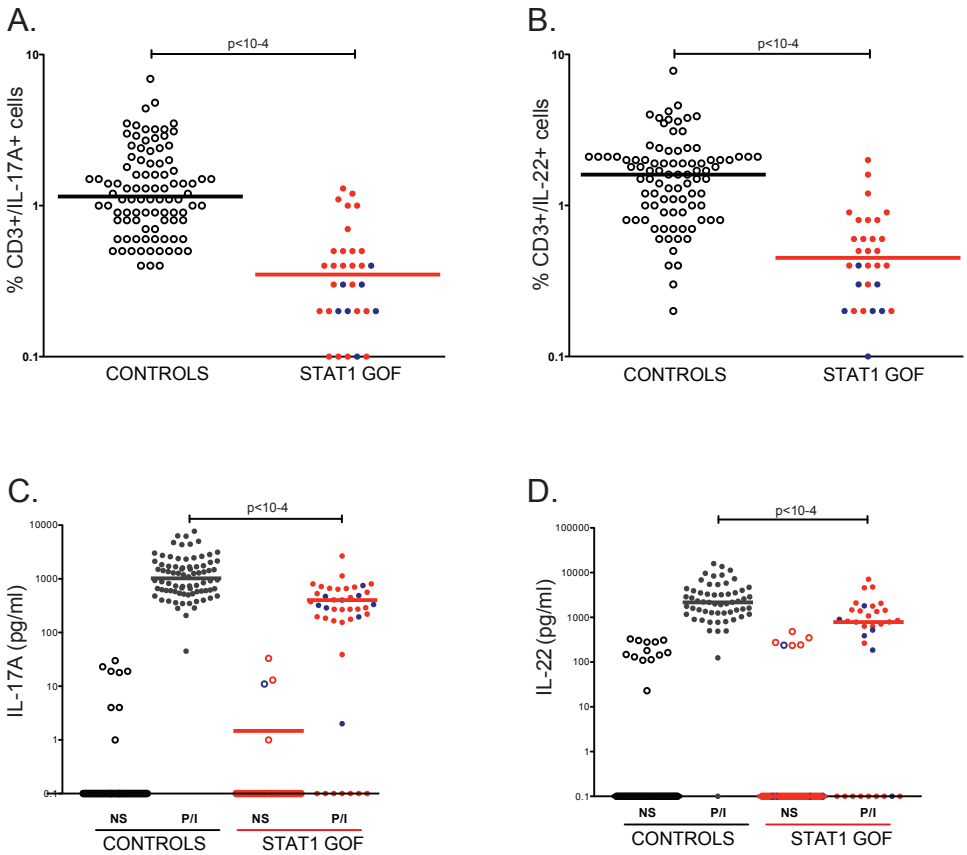

A.
p<10-4
% CD3+/IL-17A+ cells
10
1
0.1
CONTROLS
STAT1 GOF
B.
p<10-4
% CD3+/IL-22+ cells
10
1
0.1
CONTROLS
STAT1 GOF
C.
p<10-4
IL-17A (pg/ml)
10000
1000
100
10
1
0.1
NS
P/I
NS
P/I
CONTROLS
STAT1 GOF
D.
p<10-4
IL-22 (pg/ml)
100000
10000
1000
100
10
1
0.1
NS
P/I
NS
P/I
CONTROLS
STAT1 GOF

Figure 5.

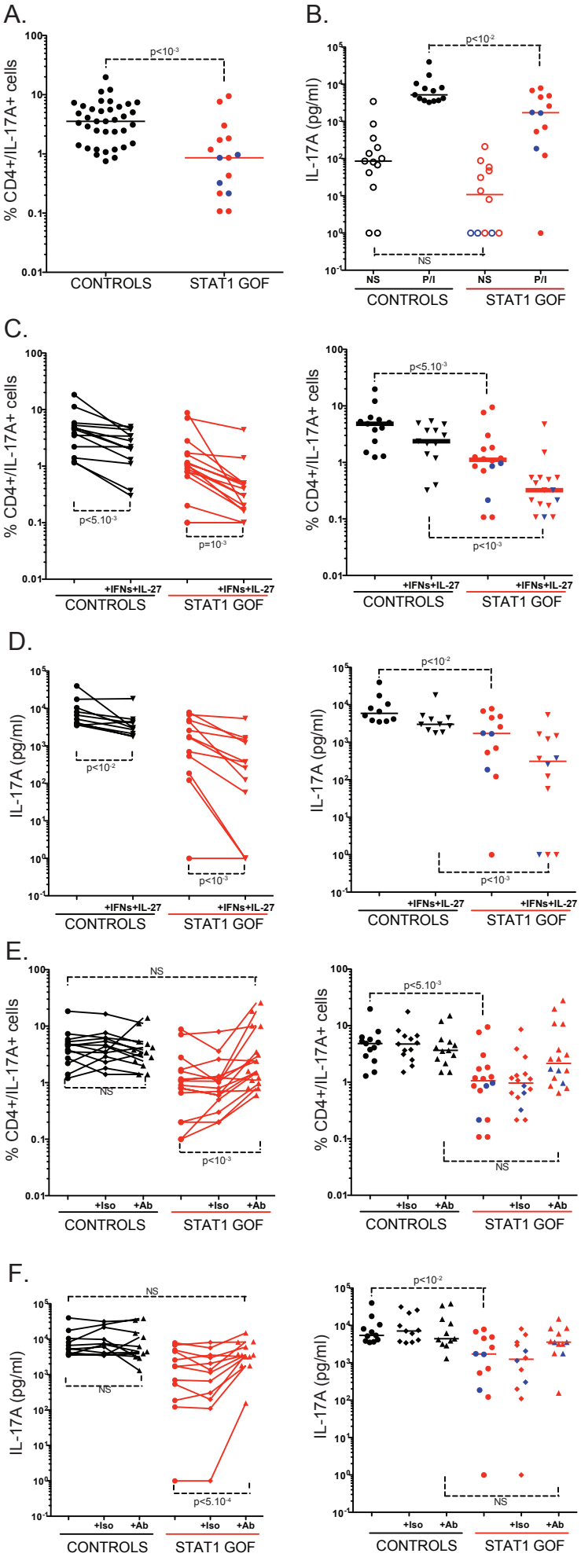

A.
B.
C.
D.
E.
F.
% CD4+/IL-17A+ cells
IL-17A (pg/ml)
CONTROLS
STAT1 GOF
NS
P/I
+IFNs+IL-27
+Iso
+Ab
p<10-3
p<10-2
p<5.10-3
p=10-3
p<5.10-4

Table 1

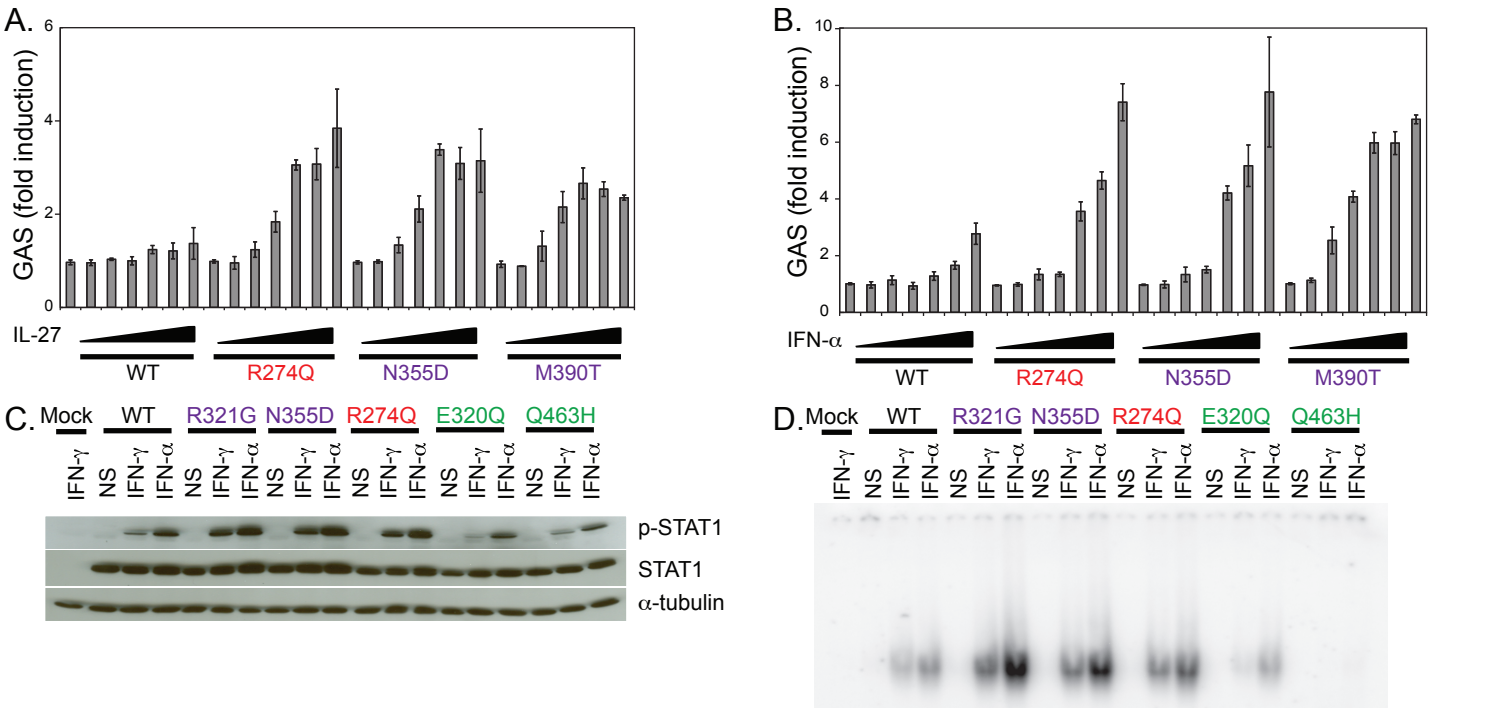
Patient	Age at presentation	Origin	Clinical features of CMC	Cause of death (age)	Autoimmunity	Genotype
A-II-1	-	Germany	Skin, oral cavity, esophagus	Lung abcess	Thyroid	-
A-III-1	<1 yr	Germany	Skin, nails, oral cavity, esophagus, oropharynx		None	D171N/WT
B-III-1	<1 yr	USA	Oral cavity, oesophagus		-	A267V/WT
C-III-6	Birth	Italy	Skin, oropharynx	Hepatitis (6 yr)	None	-
C-III-7	2 yr	Italy	Skin, nails, oral cavity		positive AAN	Q167P/WT
D-III-1	18 mo	Africa	Nails, oral cavity		None	A267V/WT
E-III-1	1 yr	France	Skin, oral cavity, esophagus		None	R274Q/WT
F-III-1	7 yr	Morocco	Skin, nails, oral cavity		None	R274W/WT
G-III-1	20 yr	France	Skin, oral cavity		None	A267V/WT
H-I-1	10 yr	France	Oral cavity	Accident (>40yr)	None	-
H-II-1	1 mo	France	Skin, nails, oral cavity		Hepatitis	R274W/WT
I-II-1	1 yr	Turkey	Oral cavity, nails		None	R274W/WT
J-II-1	12 mo	France	Skin, nails, oral cavity, genital mucosae		None	Y287H/WT
K-I-2	1 yr	France	Skin, nails, oral cavity, esophagus		None	R274Q/WT
K-II-1	2 mo	France	Skin, oral cavity, genital mucosae		None	R274Q/WT
L-II-1	Birth	United States	Skin, nails, oral cavity, esophagus		None	P293T/WT
M-I-2	9 mo	United States	Skin, nail, oral cavity, esophagus, genital		None	R274W/WT
M-II-1	3 yr	United States	Skin, nail, oral cavity, esophagus		None	R274W/WT
N-II-1	7 yr	Tunisia	Skin,, oral caity, esophagus		None	L163R/WT
O-II-1	11 mo	Chili	Skin, oral cavity		None	A267V/WT
O-I-2	2 yr	Chili	Skin, nails, oral cavity		None	-
P-II-1	3 yr	Chili	Skin, oral cavity, genital mucosae		Thyroid, colitis	R274Q/WT
Q-II-1	2 yr	Morocco	Skin, oral cavity, Genital mucosa		Hepatitis	D168E/WT
R-II-1	3 yr	Germany	Oral cavity, Nails, Skin, esophagus	Subarachnoid hemorrage on aneurysm (25 yr)	None	R274W/WT
S-II-1	-	Germany	Skin,nails, oral cavity, oropharynx,		None	P293L/WT
T-I-2	childhood	Germany	Oral cavity, nails		None	R274Q/WT
T-II-1	11 yr	Germany	Oral cavity			?
T-II-2	10 yr	Germany	Oral cavity		None	R274Q/WT
U-II-2	64yr	Germany	Skin, oral cavity		None	C324R/WT
U-III-1	32yr	Germany	Skin, oral cavity		None	C324R/WT
V-I-1		France	-		-	-
V-II-3	1 mo	France	Oral cavity, genital mucosae		None	N355D/WT
V-III-1	12 mo	France	Oral cavity, genital mucosae		None	-
V-III-2	1 mo	France	Oral cavity, genital mucosae		None	N355D/WT
W-II-1	4 mo	Germany	Oral cavity		Thyroid	K388E/WT
W-III-1	2 yr	Germany	Skin, oral cavity		None	K388E/WT
W-III-2	1 yr	Germany	Oral cavity		None	K388E/WT
X-III-3	1 mo	Peru	Skin, oral cavity		None	N397D/WT
Y-I-1	-	France	Oropharynx		-	E353K/WT
Y-II-1	3 yr	France	Skin, oral cavity		-	E353K/WT
Z-III-2	2 mo	Hungary	Skin, oral Cavity		positive AAN	R321G/WT
AA-III-1	18 mo	France	Oral cavity, nails, genital mucosae		None	L351F/WT
AB-II-1	3 mo	France	Oral cavity, nails,esophagus, genital mucosae		Crohn's disease	T385M/WT
AC-II-1	18 mo	USA	Skin, oral cavity		None	T385M/WT
AD-II-1	10 yr	Germany	Skin, oral cavity, nails		None	N357D/WT
AE-II-1	31yr	Germany	Skin, oral cavity, nails		None	N357D/WT
AF-II-1	3 mo	France	Skin, oral cavity, genital mucosae		Photosensibility, positive AAN	K388E/WT
AG-II-1	2 yr	Japan	Skin, oral cavity	Complication following stem cell transplantation (30 yr)	Thyroid	M390T/WT
AH-I-1	<1 yr	Thailand	Oral cavity	Histoplasma, cirrhosis on C hepatitis (>40yr)	-	K388E/WT
AH-II-1	10 mo	Thailand	Oral cavity		None	K388E/WT
AH-II-2	1 yr	Thailand	Oral cavity		Thyroid	K388E/WT
AI-II-1	5 yr	Ukraine	Oral cavity, skin, scalp, esophagus, genital mucosa, nails		Antiphospholipid antibodies, Anti-DNA	T385M/WT
AJ-II-1	3 yr	Germany	Skin, nails, oral cavity		None	P329L/WT
AK-I-2	-	USA	Skin, oral cavity	Hepatitis (>40yr)	Hepatitis	-
AK-II-1	-	USA	Oral cavity, skin, scalp, esophagus, nails		None	R321S/WT
AK-II-2	-	USA	Skin, oral cavity		Thyroid	R321S/WT
AK-II-3	-	USA	Skin, oral cavity		Diabetes mellitus	R321S/WT
AL-I-2		France	Oral cavity, esophagus		None	T385M/WT

None of the patients displays auto-antibodies against IL-17A, IL-17F and IL-22; .

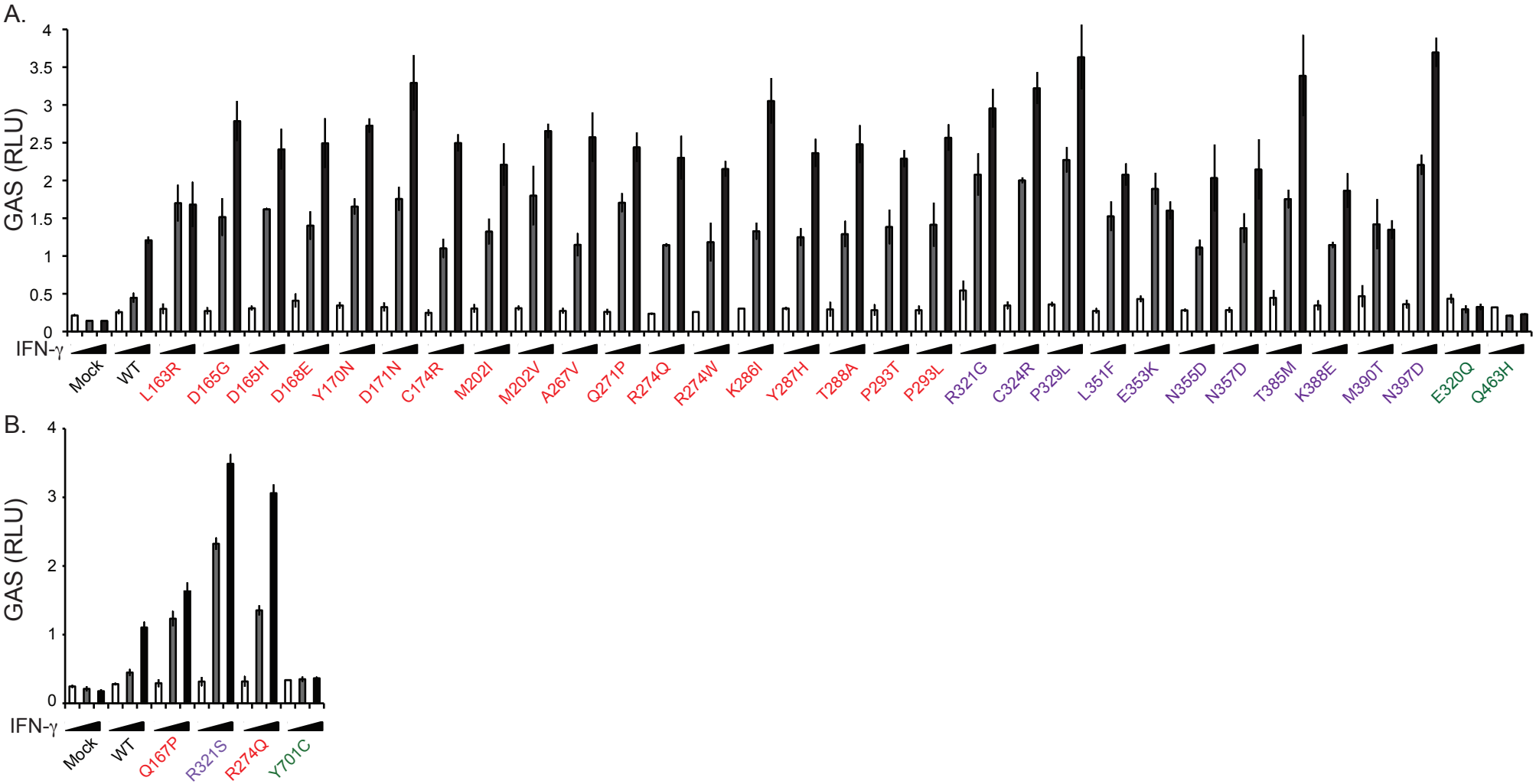
- unknown

* Firinu D, Massidda O, Lorrai MM, Serusi L, Peralta M, Barca MP, Serra P, Manconi PE (2011) Successful treatment of chronic mucocutaneous candidiasis caused by azole-resistant Candida albicans with posaconazole. Clin Dev Immunol 2011: 283239

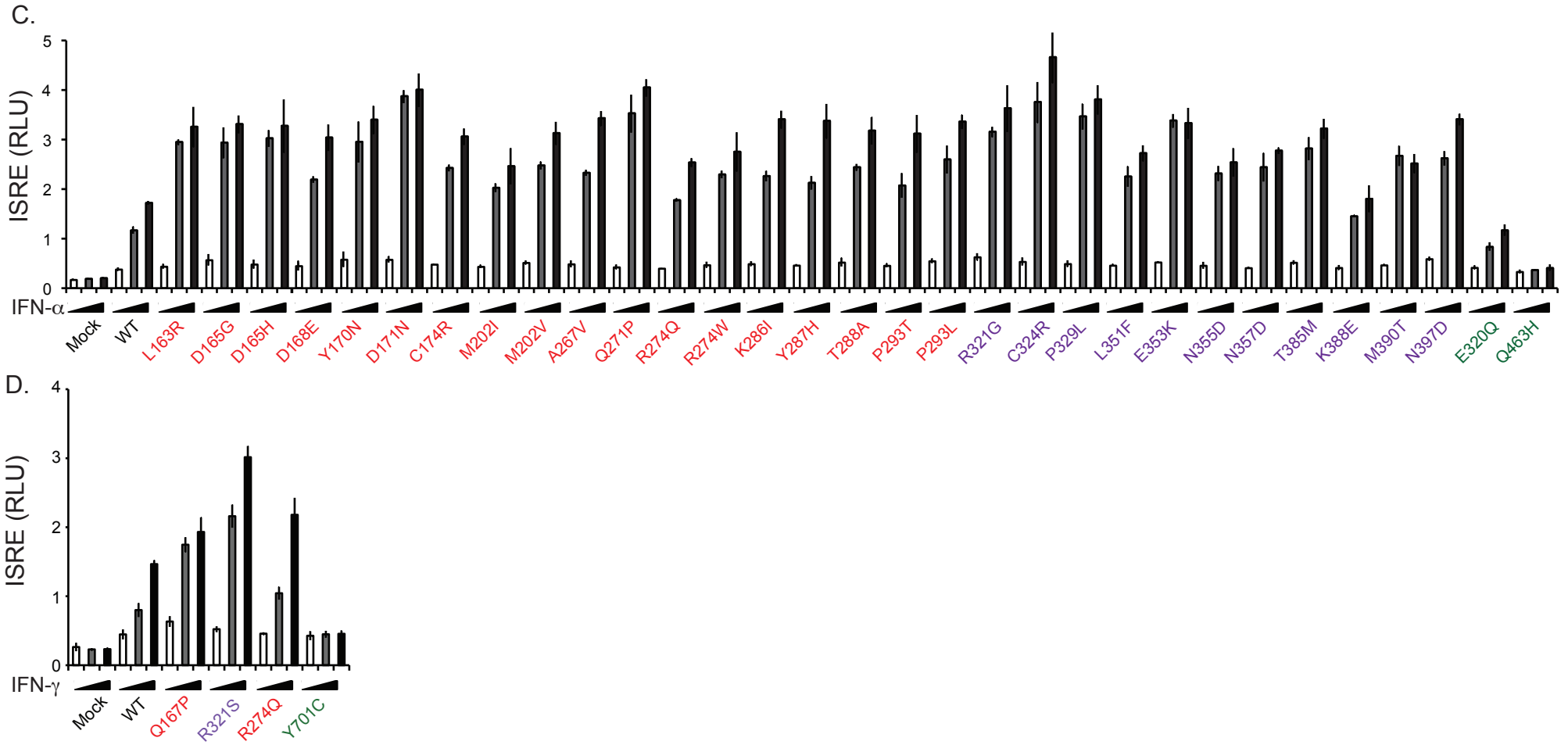
Supplementary Figure 1



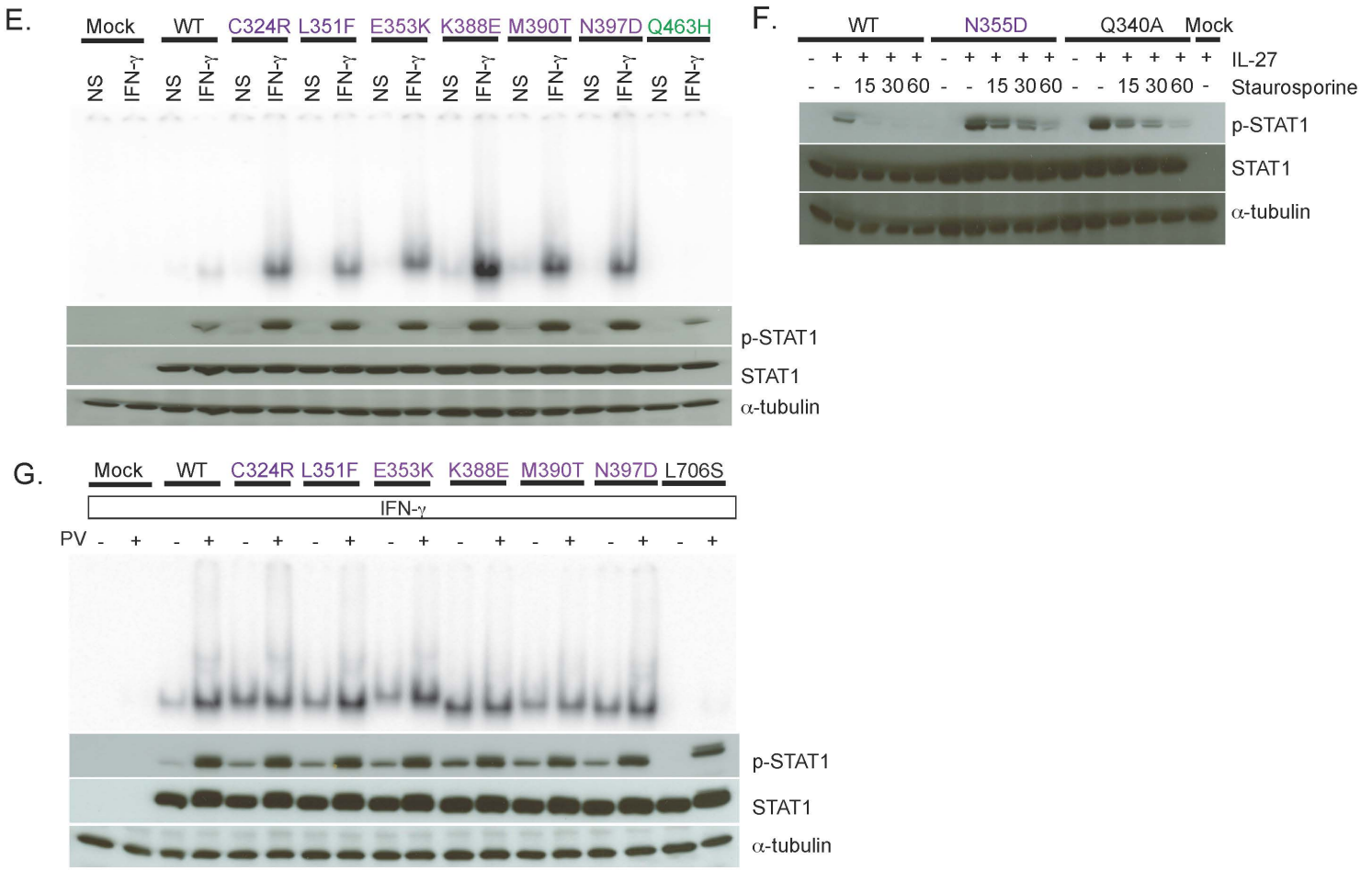
Supplementary Figure 2



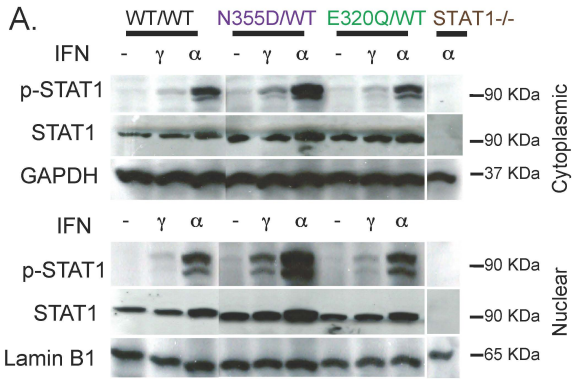
Supplementary Figure 2

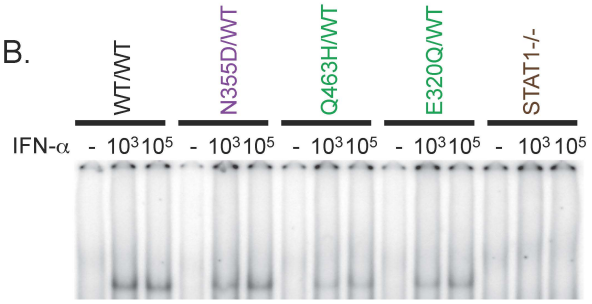


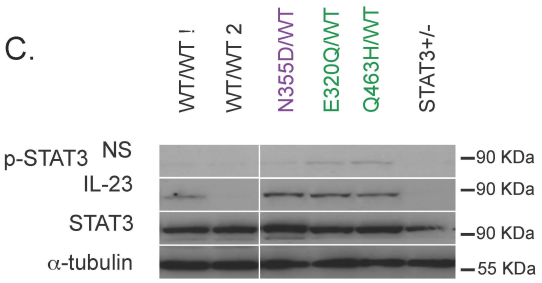
Supplementary Figure 2



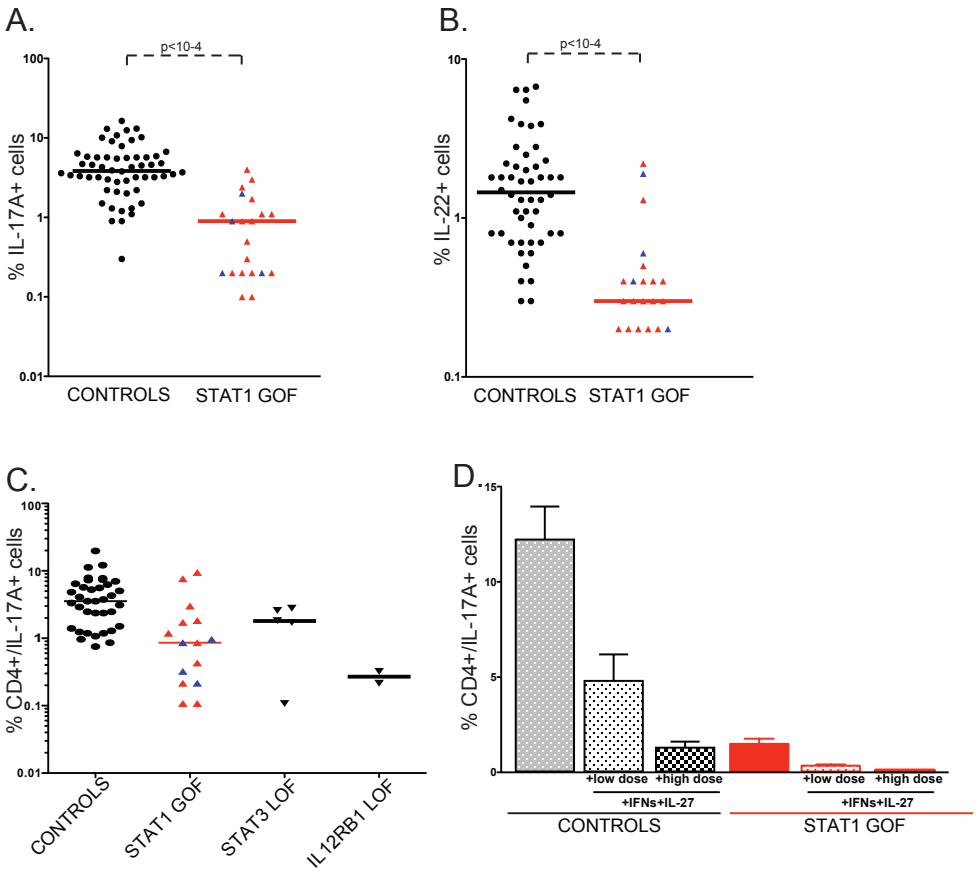
Supplementary Figure 3



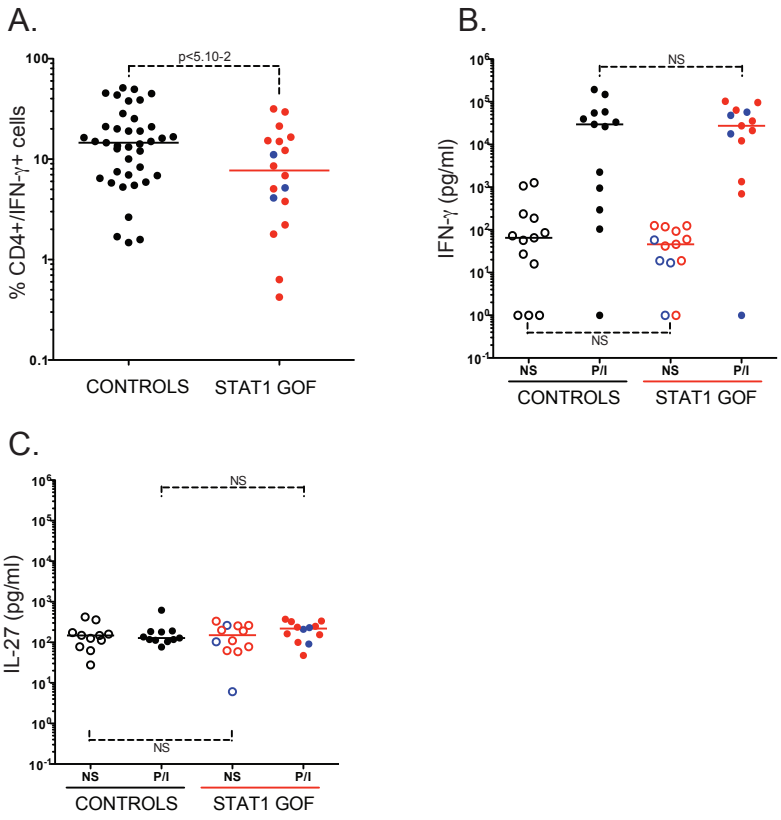




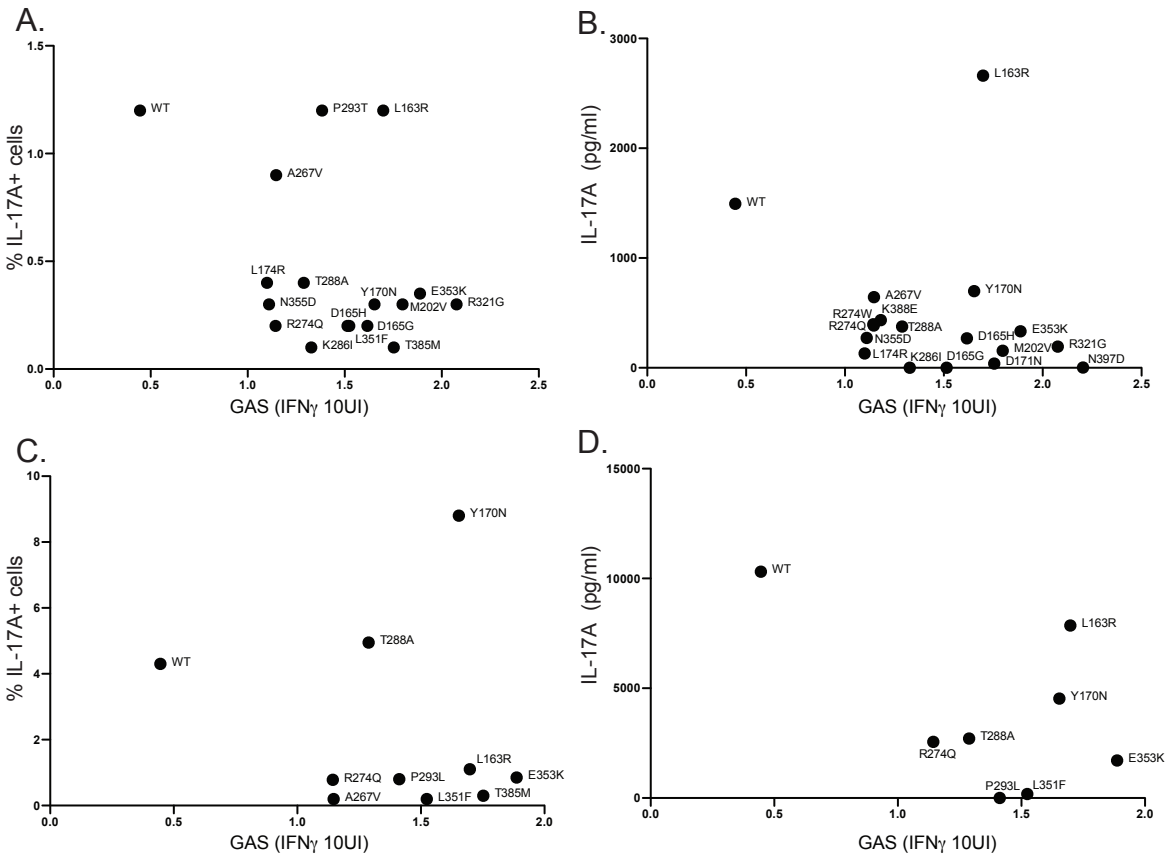
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Suppl. Table 1

	cdna position	codons	existing variation	Location	Allele	Protein pos.	AA change	**Sift**	**PPH2**	**Mass**	**Condel**	Condel lbl
1	876	cTg/cGg	-	2:191864405	C	163	L/R	**0**	**0.997**	**2.83**	**0.966**	**deleterious**
2	888	cAa/cCa	-	2:191864393	G	167	Q/P	**0**	**1**	**2.87**	**1**	**deleterious**
3	892	gaT/gaG	-	2:191864389	C	168	D/E	**0.11**	**0.958**	**1.985**	**0.412**	**possibly**
4	899	Gac/Aac	-	2:191864382	T	171	D/N	**0.01**	**0.996**	**2.775**	**0.867**	**deleterious**
5	1247	Tac/Cac	-	2:191859872	G	287	Y/H	**0**	**0.086**	**1.32**	**0.006**	**neutral**
6	1265	Cct/Act	-	2:191859854	T	293	P/T	**0**	**0.994**	**2.765**	**0.952**	**deleterious**
7	1266	cCt/cTt	-	2:191859853	A	293	P/L	**0.01**	**1**	**2.42**	**0.905**	**deleterious**
8	1349	Aga/Gga	-	2:191856030	C	321	R/G	**0.01**	**1**	**2.65**	**0.905**	**deleterious**
9	1351	agA/agT	-	2:191856032	A	321	R/S	**0.01**	**1**	**2.65**	**0.905**	**deleterious**
10	1358	Tgc/Cgc	-	2:191856021	G	324	C/R	**0**	**1**	**2.43**	**1**	**deleterious**
11	1374	cCt/cTt	-	2:191856005	A	329	P/L	**0.36**	**0.809**	**1.235**	**0.308**	**neutral**
12	1441	ttG/ttT	-	2:191854385	A	351	L/F	**0.33**	**0.997**	**1.86**	**0.478**	**deleterious**
13	1445	Gag/Aag	-	2:191854381	T	353	E/K	**0.05**	**0.998**	**1.355**	**0.82**	**deleterious**
14	1451	Aat/Gat	-	2:191854375	C	355	N/D	**0.15**	**0.997**	**2.24**	**0.478**	**deleterious**
15	1457	Aat/Gat	-	2:191854369	C	357	N/D	**0.03**	**0.266**	**1.895**	**0.04**	**neutral**
16	1542	aCg/aTg	-	2:191851647	A	385	T/M	**0**	**1**	**2.815**	**1**	**deleterious**
17	1550	Aaa/Gaa	-	2:191851639	C	388	K/E	**0**	**0.995**	**1.9**	**0.958**	**deleterious**
18	1557	aTg/aCg	-	2:191851632	G	390	M/T	**0**	**0.74**	**2.69**	**0.771**	**deleterious**
19	1577	Aat/Gat	-	2:191851612	C	397	N/D	**0.03**	**0.603**	**2.36**	**0.574**	**deleterious**

ANNEX

Publication 4

Homozygous CARD9 mutations in patients with invasive dermatophytic disease

Lanternier F*, Pathan S*, Vincent Q, **Liu L**, Cypowij S, Prando C, Migaud M, Taibi L, Ammar-Khodja A, Stambouli OB, Guellil B, Merad-Boudia A, Jacobs F, Goffard JC, Shepers K, Marmol V, Bachelez H, Michel L, Lefranc G, Fraitag S, Bougnoux ME, Lortholary O, Abel L, Jean-Laurent Casanova JD*, Picard C*, Grimbacher B* and Pue1 A*

* These autors contributed equally to this study.

N Engl J Med, in press

Homozygous *CARD9* mutations in patients with invasive dermatophytic disease

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Abstract

Dermatophytic disease is an invasive, sometimes life-threatening, fungal infection caused by dermatophytes with extensive cutaneous and subcutaneous tissue involvement. Dissemination to the lymph nodes is frequent, and the central nervous system is occasionally affected. This rare condition has mostly been reported in North African consanguineous multiplex families, strongly suggesting a Mendelian genetic etiology. Autosomal recessive CARD9 deficiency has been described in a multiplex consanguineous Iranian family, the affected members of which display mucocutaneous disease and life-threatening meningitis caused by *Candida* species and skin infections with dermatophytes. We investigated 13 patients with invasive dermatophytic disease from six unrelated Algerian and Moroccan families. Morbidity rates were high, with extensive erythemato-squamous, ulcerative and/or nodular lesions, organ involvement (bone or digestive tract) and lymph node dissemination despite the use of recent antifungal drugs. Four of the 13 patients died. No other severe infections were reported in the surviving patients, who were aged 40 to 75 years. We sequenced *CARD9* in 10 of the 13 patients. Eight Algerian patients from five unrelated families had a homozygous Q289X *CARD9* allele, probably due to a founder effect. Two affected Moroccan siblings were homozygous for the R101C *CARD9* allele. Both these alleles are rare variants that were not found in more than 3,500 healthy individuals tested, including 100 individuals of Northern African descent. The familial segregation of these alleles was consistent with autosomal recessive inheritance and complete clinical penetrance. Invasive dermatophytic disease may thus be caused by autosomal recessive CARD9 deficiency. Inborn errors of immunity should therefore be considered in otherwise healthy patients with unexplained severe fungal disease.

Introduction

Dermatophytic disease is a rare, invasive, sometimes life-threatening, fungal infection caused by dermatophytes (1). These filamentous fungi (mostly *Trichophyton rubrum,* but also *T. mentagrophytes, T. interdigitale* and *T. violaceum*) are ubiquitous and usually cause benign disease, in which the infection is limited to keratinized tissues, such as the nails, skin, groin, toes and hair, leading to onychomycosis, tinea corporis, cruris, pedis or capitis (2). Such superficial dermatophytic infections are frequent, onychomycosis having a prevalence of about 2.6 to 13% in North Africa and Europe (3). By contrast, dermatophytic disease is characterized by the invasion of deeper tissues (dermis, hypodermis), extensive skin, hair and nail involvement and visceral dissemination (lymph nodes, central nervous system), as first described by Hadida and Schousboe in 1959 (1). Forty-five cases have been reported to date in individuals from North Africa (Algeria, Morocco and Tunisia) (1, 4-25). Twenty-four patients belonged to consanguineous families, including 5 sporadic cases and 19 familial cases from 8 multiplex families. The remaining 21 cases belonged to families not reported to be consanguineous, including 14 sporadic cases and 7 familial cases from 3 families. This is strongly suggesting that predisposition to dermatophytic disease is inherited as an autosomal recessive trait. In addition, nineteen sporadic non consanguineous cases have also been reported in England, Russia, Denmark, Mexico, Brazil, the United States, and Japan (8, 26-34).

Interest in genetic susceptibility to fungal diseases in otherwise healthy patients has increased in recent years. Inborn errors of IL-17 immunity have been reported to underlie chronic mucocutaneous candidiasis (CMC) (35-38). Autoantibodies against IL-17 cytokines underlie CMC in autoimmune polyendocrinopathy syndrome type I (APS-I) (39, 40). Moreover, patients with the autosomal dominant (AD) hyper IgE syndrome due to

heterozygous mutations in *STAT3* display impaired development of IL-17-producing T cells (41-45). Finally, loss-of-function mutations in *IL17F* and *IL17RA* (35), and gain-of-function mutations in *STAT1* (36, 37) have all been associated with impaired IL-17-mediated immunity and CMC disease (46). Invasive fungal infections have long been known to affect some patients with primary immunodeficiencies (PIDs). Patients with chronic granulomatous disease (CGD) and an impaired oxidative burst (47) are susceptible to multiple bacterial and fungal infections, including invasive *Aspergillus* spp. infections in particular. Children with severe congenital neutropenia are prone to various invasive fungal diseases (48). Patients with IL-12Rβ1 or IFN-γR1 deficiencies or with X-linked hyper IgM are susceptible to disseminated infections with dimorphic fungi (47, 49-51). Patients with autosomal recessive (AR) CARD9 deficiency, reported to date in only one multiplex Iranian kindred, develop *Candida* spp. meningitis, in addition to CMC and cutaneous dermatophytosis (52). However, dermatophytic disease has not been reported in any of these disorders. We therefore used a candidate gene approach, including the sequencing of *CARD9*, to investigate 13 patients with dermatophytic disease from six individually consanguineous but unrelated kindreds from Algeria and Morocco.

Results

Case summary

The characteristics of the patients with dermatophytic disease studied are reported in Table 2. Briefly, we report 13 patients: nine men and four women. The median age of the patients was 40 [28-75] years. Eleven of the patients were from Algeria and two were from Morocco. The first symptoms of dermatophytosis appeared during childhood or adolescence, at a median age of eight years [2-21]. Initial symptoms were mostly recurrent and severe tinea capitis was reported for 10 patients, tinea corporis for seven and onychomycosis for six patients. Skin lesions subsequently included extensive erythemato-squamous lesions, nodular subcutaneous or ulcerative fistulizing infiltrations. Seven patients had enlarged lymph nodes, one presented central nervous system involvement, and two had local infiltration of the bone or digestive tract, respectively. *T. violaceum* was the dermatophyte species most frequently isolated from lesions. It was recovered from six patients. The only associated infectious condition was mycologically confirmed thrush in four patients. These patients presented no other superficial or invasive *Candida* spp. infection. Four patients died from their disease at the ages of 28, 29, 37 and 39 years, due to disease progression or superinfection.

Case reports

We report 13 patients with dermatophytic disease (9 men and 4 women) from six unrelated kindreds. The six families originated from Morocco (1 family) and Algeria (5 families) (Table 1) and the characteristics of the patients are reported in Table 2.

Kindred A: Proband P1 (A.II.1, Figure 1) was born to consanguineous Algerian parents from Tlemcen and developed dermatophytic disease at the age of six years. Initial clinical presentation was as tinea corporis, tinea capitis and onychomycosis with

pachyonychia (Figure 2A, A-D) (4). Non insulin-dependent diabetes mellitus was diagnosed at the age of 50 years. At this age, blood counts revealed eosinophilia (counts of up to 1700/mm^3) and cultures of skin samples from multiple sites were positive for *Trychophyton violaceum*. When the patient was 52 years old, the erythematous lesions on the skin extended, with severe itching, and a large, soft, subcutaneous polylobate tumor (10 x 15 cm) appeared in the patient's left armpit (Figure 2A, D). P1 also presented large axillary, submaxillary (4 cm) and mesenteric lymphadenopathies. Histological examination of the axillary lymphadenopathy revealed the presence of hyphae within a necrotic granuloma. T (CD4$^+$, CD8$^+$), B and NK lymphocyte subsets were normal. Treatment with a combination of griseofulvin and econazole was not effective. P1 is now 75 years old and has been treated with itraconazole since June 2011, resulting in a decrease in the size of the lesions. No other severe infections were reported.

One of the cousins of P1 (P2, A.III.6, Figure 1) (56, 57) also presented dermatophytic disease beginning with onychomycosis at the age of two years. He then developed recurrent tinea capitis and corporis and lymphadenopathy at the age of eight years and mycologically confirmed oral thrush at the age of nine years. At 25 years of age, P2 presented erythroderma, cutaneous nodules (several of which were ulcerated), alopecia and onychodystrophy. He also had iliac and inguinal, axillary and cervical fistulizing lymphadenopathies (Figure 2A, E). *T. violaceum* was isolated from skin and lymph nodes. Histological examination of skin samples revealed the presence of hyphae within a necrotizing granuloma. Eosinophilia (2700/mm^3) was detected at the age of 25 years. P2 subsequently developed seizures, leading to the detection of three cerebral abscesses on CT scan (Figure 2A, F). Despite itraconazole treatment, the cerebral and skin lesions worsened and the patient died from septicemia at the age of 29 years (56, 57). No other severe infections were reported.

Two of the siblings of P2 suffered from dermatophytic infections. One brother (A.III.11, Figure 1) presented tinea capitis and onychomycosis with *Trichopyton rubrum* in childhood, before the age of 12 years, and a sister (A.III.7, Figure 1) had interdigital intertrigo and onychomycosis caused by *T. rubrum* at the age of 26 years. No follow-up data are available for these two siblings.

Kindred B: Patient P3 (B.II.6, Figure 1) is a woman from a consanguineous Algerian family from Tlemcen. Her dermatophytic disease began with recurrent tinea capitis and extensive tinea corporis at the age of nine years (58). She was immunized with BCG with no adverse effects. Following a course of treatment with systemic corticosteroids at the age of 12 years, she developed a severe skin infection with general thickening, lichenification, squamous areas, pruritus, multiple erythematous nodules, palmo-plantar keratotic lesions (Figure 2A, G), severe nail involvement with onychogryphosis, and squamous scalp. P3 also developed multiple lymphadenopathies with fistula formation (Figure 2A, H), worsening between the ages of 12 and 17 years. *T. rubrum* grew from cultures of skin, scalp, nails and lymph nodes taken from the patient at the age of 17 years. Histological examination of lymph nodes showed necrotizing granuloma with eosinophils and hyphae, stained with Positive Acid Schiff reagent, in giant multinucleate cells. T ($CD4^+$, $CD8^+$), B and NK lymphocyte subsets were normal. When the patient was 17 years old, treatment with griseofulvin at a dose of 1 g/day was introduced. This treatment was continued for two years and resulted in a clear improvement. The treatment was then stopped, but dermatophytic disease relapsed. At the age of 35 years, P3 developed insulin-dependent diabetes mellitus. She is now 40 years old. Itraconazole treatment was initiated in June 2011 and again led to a reduction of the lesions and itching. No other severe infections were reported. Neither the patient's parents nor her siblings has ever suffered from a dermatophytic infection.

Kindred C: The proband, P4 (C.II.1, Figure 1), is from a consanguineous Algerian family from Algiers. His dermatophytic disease has been described elsewhere (7). He was immunized with BCG with no adverse effects. He began to suffer from recurrent tinea capitis (Figure 2A, I) and tinea corporis at the age of eight years. He then developed extensive foot and hand onychomycosis and glabrous skin lesions with lichenification (Figure 2A, I-L). He also had recurrent *C. albicans* thrush. *T. violaceum* was cultured from skin lesions after seven months of growth. IgE levels were high (1300 U/ml). Griseofulvin treatment was initiated when the patient was 17 years old and led to some improvement. However, chronic residual lesions remained and relapses occurred whenever the treatment was stopped. P4 is now 56 years old, is still treated with griseofulvine and has residual skin lesions. No other severe infections were reported.

His brother (P5, C.II.5, Figure 1) (59) first showed signs of the disease at the age of eight years, with extensive tinea capitis and onychomycosis. He then presented extensive keratotic and ichthyotic lesions, disseminated papular nodules alopecia, pachyonychia and onycholysis. Several subcutaneous abscesses and all peripheral enlarged lymph nodes fistulized. At the age of 15 years, P5 had an eosinophil count of 2600/mm^3 and high IgE levels (1300 U/ml). *T. violaceum* was isolated from skin lesions (after five months of growth). P5 also had recurrent thrush caused by *C. albicans*. Griseofulvin treatment was initiated when the patient was 15 years old. No other severe infections were reported. Despite initial improvement, the patient's disease worsened and he died from disseminated dermatophytic disease at 34 years of age (59).

The sister of these two patients (P6, C.II.11, Figure 1) is currently 41 years old. At the age of eight years, she presented chronic onychomycosis of all nails due to *T. violaceum*. She

was treated with griseofulvin and presented no other infections. Neither the parents nor the other siblings had any dermatophytic infections.

Kindred D: The proband, P7 (D.II.6, Figure 1), from a consanguineous Algerian family from Constantine, presented ulcerative and nodular lesions of the left thigh (Figure 2A, M) and scalp (Figure 2A, N) at the age of 19 years. He had been immunized with BCG with no adverse effect. He developed recurrent tinea capitis, onychomycosis of the hands and feet and enlargement of the cervical lymph nodes, at the age of 39 years. Skin biopsy at the age of 20 years provided evidence of a hyperkeratotic epithelium with granuloma. Tuberculosis was initially suspected and the patient was given antimycobacterial therapy for nine months, without improvement. A second skin biopsy at the age of 40 years revealed the presence of hyphae within the granuloma. Skin scrapings also demonstrated the presence of hyphae. However, no dermatophytes could be grown from the lesions in culture. At the age of 40 years, P7 was treated with griseofulvin and fluconazole, with temporary improvement, and relapse occurred when the antifungal drugs were withdrawn. The patient is now 43 years old, treated with griseofulvin and fluconazole and has residual, but stable skin lesions. No other severe infections were reported.

His brother (P8, D.II.7, Figure 1), who is now 40 years old, has also had dermatophytic disease since the age of 21 years, with extensive ulcerating skin lesions on the face, scalp and perineum. He was immunized with BCG with no adverse effect. He was treated with griseofulvin between the ages of 21 and 27 years and then with fluconazole. The scalp and face lesions resolved and the perineal lesions improved. When fluconazole treatment was stopped, the perineal lesions enlarged to 20 centimeters in diameter and caused stenosis (Figure 2A, O). Surgery, with colostomy, was therefore required and terbinafine treatment was initiated in September 2010. P8 also developed extensive pityriasis versicolor,

onychomycosis and inguinal lymph node enlargement. Hyphae were found within necrotizing granulomas in skin lesions. A clear improvement was observed after one month of treatment with itraconazole and terbinafine (Figure 2A, P). No other severe infections were reported.

The brother of P7 and P8 (P9, D.II.4, Figure 1) died from dermatophytic disease, with pseudotumoral and ulcerating lesions on his face, at the age of 28 years. Neither the parents of these patients nor the other siblings suffered from dermatophytic infection.

Kindred E: The proband, P10 (E.II.7, Figure 1), from another unrelated consanguineous family from Tlemcen in Algeria, developed recurrent tinea in childhood and was subsequently diagnosed with dermatophytic disease. At the age of 27 years, he presented cutaneous erythemato-squamous warty lesions with onychomycosis and giant palmo-plantar horns (8 cm high) with onychogryphosis (60) (Figure 2A, Q-T). He had eosinophilia (550/mm^3) at the age of 29 years. *T. violaceum* was isolated from the skin and nails. Skin biopsy showed acanthosis and hyperkeratosis of the epidermis. The stratum corneum and dermis were invaded by hyphae and lymphoid granulomas. The patient improved on griseofulvin, but a relapse occurred and he died from septicemia at the age of 39 years. No other severe infections were reported.

His sister (P11, E.II.10, Figure 1), who is currently 37 years old, has suffered from chronic onychomycosis since childhood. Neither their parents nor any other siblings have been reported to suffer from dermatophytic infection.

Kindred F: The proband, P12 (F.II.6, Figure 1), was born in Belgium, but is from a consanguineous family originating from Tangier in Morocco. He presented recurrent thrush and tinea during childhood. At the age of 16 years, squamous hyperkeratosic skin lesions appeared on his left foot. These lesions worsened at the age of 35 years, with vegetative and

ulcerating lesions extending to both feet, calves and the left thigh (Figure 2A, U-V). Lesions were associated with left inguinal lymphadenopathy, squamous pigmented lesions of the groin and left foot onychomycosis. X rays suggested osteolysis of the left first and second toes and MRI revealed soft tissue infiltration (27 mm) (Figure 2B, W-Y). P12 had no other severe infection. Hypereosinophilia (up to 1500/mm^3) was observed from the age of 35 years onwards and IgE levels were high, at up to 1741 kIU/ml. T ($CD4^+$, $CD8^+$), B and NK lymphocyte subsets were normal. A skin biopsy carried out when P12 was 35 years old showed a hyperplastic epidermis, with parakeratosis and some microabscesses. The dermis was infiltrated with a tuberculoid granuloma and numerous eosinophils. PAS staining revealed irregular hyphae, with some branching, in the granuloma (Figure 3). Skin culture and molecular analyses were positive for *T. rubrum*. The lesions improved but a relapse occurred despite the sequential administration of terbinafine, voriconazole, posaconazole, liposomal amphotericin B and combined treatment with terbinafine, voriconazole and interferon-γ. The second toe on the patient's left foot required amputation. P12 is now 40 years old and his disease has relapsed despite voriconazole treatment. No other severe infections were reported.

The sister of P12 (P13, F.II.3) is 49 years old. She was born in Morocco and had recurrent severe tinea during childhood. As an adult, she has suffered from hand and foot onychomycosis. Neither the parents nor the other siblings have been reported to suffer from dermatophytic infections.

Identification of homozygous nonsense or missense *CARD9* mutations

We investigated 10 of the 13 patients with dermatophytic disease, for whom genetic material was available, by a candidate gene approach. We first sequenced *CARD9* by Sanger sequencing and found homozygous mutations in *CARD9* in all 10 patients. The eight

Algerian patients had a homozygous *c.C865T* mutation in exon 6, resulting in a premature termination codon in position 289, Q289X (Figure 4), in the region encoding the coiled-coil domain of CARD9 (Figure 1B). The two patients from the Moroccan kindred had a homozygous *CARD9* missense mutation, *c.C301T,* in exon 3, resulting in the replacement of the arginine residue in position 101 with a cysteine residue (R101C). This amino-acid substitution is located only a few amino acids after the end of the CARD domain (Figure 4). Finally, all healthy members of the six kindreds were found to be either homozygous for the wild-type allele or heterozygous for one of the two mutations. The segregation of the two mutations in six kindreds was consistent with an autosomal recessive (AR) CARD9 deficiency with complete clinical penetrance. These two mutations in patients with dermatophytic disease are different from the Q295X mutation previously reported in an Iranian kindred with CARD9 deficiency (52). The missense and nonsense mutations reported here were not found in any of the various public databases searched (HGMD, Ensembl and 1000 Genomes,) or in our in-house whole-exome sequencing database (> 1000 exomes). We also sequenced 1,052 controls from the CEPH-HGD panel, 33 controls from Algeria and 70 controls from Morocco, in whom we found neither of the two variants, thus excluding the possibility that the Q289X and R101C variants are polymorphisms. Polyphen 2 (61) predicted the missense mutation to be probably damaging (with the highest possible score of 1).

Founder-effect analysis

All eight Algerian patients harbored the same previously unknown homozygous premature termination codon (Q289X), suggesting a possible founder effect (53, 55, 62-64). An analysis of Affymetrix 250K Nsp Array data showed that patients carrying the Q289X variant had a common homozygous haplotype surrounding the *CARD9* gene (Figure 5). The

largest common haplotype upstream from the mutation was identified in patients P3 and P1, and it encompassed 1.2 megabases (corresponding to 33 SNPs). The largest common haplotype downstream from the mutation was identified in patients P8 and P4, and it encompassed 1.6 megabases (29 SNPs). The ESTIAGE method was used to estimate the age of the MRCA to 26 generations (95% confidence interval [CI]: 12-58 generations). Assuming a generation time of 25 years, the MRCA of the patients therefore lived about 650 years ago (95% CI: 300-1450 years).

Discussion

We identify homozygous mutations of *CARD9* as the first genetic etiology of dermatophytic disease, thus broadening the spectrum of invasive fungal infections associated with CARD9 deficiency. Following on from the Q295X nonsense mutation previously reported in a large multiplex consanguineous Iranian family (52), we identified two new *CARD9* mutations, one of which was missense (R101C), the other being a nonsense mutation (Q289X). Patients homozygous for these deleterious alleles were found in three countries (Iran (52), Morocco, and Algeria) in which the frequency of parental consanguinity is high. The five Algerian families carried the same Q289X mutation due to a founder effect, with the most recent common ancestor living approximately 650 years ago. None of the heterozygous individuals in the kindreds studied had any symptoms, whereas all individuals homozygous for the mutated allele had symptoms, consistent with an AR mode of inheritance and complete clinical penetrance. Thus, 19 patients from 7 families in three countries, with three different alleles have now been identified. CARD9 deficiency is thus clearly not restricted to a single kindred, as initially hypothesized based on the rarity of the clinical presentation of the Iranian kindred (52). Indeed, we show that CARD9 deficiency is the main genetic etiology of dermatophytic disease. It is possible that *CARD9* mutations will be identified in other patients with dermatophytic disease (1, 4-27, 29-34, 56-58, 60, 65, 66).

The clinical signs of dermatophytic disease in the patients described here began in childhood, with recurrent and severe tinea and onycomycosis and worsened during adolescence, leading to invasive disease. Skin and lymph node biopsies provided evidence of hyphae consistent with dermatophyte infection, within epithelioid and giant cell granulomas, often associated with necrosis and eosinophil infiltration. These clinical signs are similar to those observed in tuberculosis, and the patients also presented fistulized lymph nodes and

subcutaneous abscesses. Survival was poor in the patients reported here, with four of the 13 patients studied dying between the ages of 28 and 39 years due to visceral disease or superinfections. However, no other severe infections were reported, with no mycobacterial or *Listeria* infections in particular, by contrast to what has been reported for CARD9-deficient mice, which are susceptible to *Mycobacterium tuberculosis, Candida sp.* and *Listeria monocytogenes* (67-71). The patients with *CARD9* mutations and dermatophytic disease reported here had clinical characteristics similar to those reported in previous studies (1, 4-27, 29-34, 56-58, 60, 65, 66). Disease started in childhood (11 years in previous studies vs. 8 years in our series) with severe and recurrent tinea capitis (51.7% vs. 77%) or tinea corporis (41% vs. 54%). The frequency of onychomycosis as a presenting symptom was higher in our study than in previous publications (46% vs. 7%) (72). Dissemination was frequent with lymph node (58% vs. 54% in our series) or central nervous system (9% vs. 8%) involvement (72). Bone involvement was less common with five cases reported altogether ((32, 33), this report). Histological findings are also similar, including the formation of tuberculoid granulomas containing hyphae. *T. violaceum* was the most prevalent species isolated, both in previous studies (29%) and in our series (75%). *T. violaceum* is also the species most frequently isolated from patients in North Africa (44% in Libya) (3). Two discrepancies between our findings for these patients and published results were identified: a higher frequency of thrush (31%, whereas no cases were reported among the other patients and a lower mortality (31% in our series vs. 44%), probably reflecting the use of new antifungal treatments, such as itraconazole and voriconazole in the most recently diagnosed patients.

Nineteen patients homozygous for deleterious *CARD9* alleles have now been reported and these patients present various fungal infections, including superficial and invasive fungal diseases. These findings highlight the essential role of CARD9 in the human immune responses controlling fungal infection. The molecular and cellular basis of fungal disease,

including dermatophytic disease in particular, remains unclear. A macrophage defect might account for the invasion of the dermis by dermatophytes in CARD9-deficient patients. CARD9 is an adaptor in the signaling pathway downstream from Dectin-1, Dectin-2, Mincle and, probably, other as yet unknown receptors involved in antifungal immunity (68-70, 73-78). However, it remains unclear which receptors are actually involved in immunity to dermatophytes. The diverse clinical presentations of CARD9 deficiency, ranging from dermatophytosis to *C. albicans* meningitis, suggest that multiple molecular pathways in multiple cell types are controlled by CARD9. Patients with CARD9 deficiency have previously been reported to have low proportions of interleukin (IL)-17 T cells, possibly accounting for CMC (52). Additional studies are required to characterize the CARD9-dependent pathways in both myeloid and lymphoid cells in humans, together with the other genes responsible for controlling host defense against *Candida* spp. and dermatophytes. Dermatophytic disease is associated with AR *CARD9* mutations in all families tested to date.

Methods

Patients

Thirteen patients with a history of dermatophytic disease were recruited from five hospitals (Mustapha Hospital, Algiers, Algeria; Tlemcen Hospital, Tlemcen, Algeria; Sidi Bel-Abbes Hospital, Sidi Bel-Abbes, Algeria; Necker-Enfants Malades, Paris, France and Erasme Hospital, Brussels, Belgium). Diagnosis was based on medical and family history, clinical signs, histopathology and mycology results. This study was conducted in accordance with the Helsinki Declaration. All patients provided written informed consent for participation in the study.

Molecular genetics

Genomic DNA was isolated from whole blood, by a phenol/chloroform extraction method, with an iPrep extraction kit (from Invitrogen) or with the Gentra Puregene Blood kit (Qiagen, UK). *CARD9* was amplified with specific primers (PCR amplification conditions and primer sequences are available upon request). PCR products were analyzed by electrophoresis in 1% agarose gels, sequenced with the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA), and analyzed on an ABI Prism 3700 (Applied Biosystems, Foster City, CA).

Most recent common ancestor of Q289X carriers

Founder-effect analysis was carried out on a subset of four available, apparently unrelated patients (two from Tlemcen (P1, P3), one from Algiers (P4), one from Constantine (P8)) bearing the same homozygous Q289X CARD9 mutation. Genotypes were obtained for > 250,000 single-nucleotide polymorphisms (SNPs) on the Affymetrix GeneChip Human Mapping 250K Nsp Array. SNPs with a 100% call rate were scanned for continuous stretches of

homozygosity upstream and downstream from the *CARD9* locus on chromosome 9q34. Pairwise comparisons within each mutation group revealed the limits of the longest shared haplotype and the positions of subsequent recombination break points. The likelihood-based ESTIAGE method was used to estimate the most recent common ancestor (MRCA) for each mutation from the observed shared haplotypes. Recombination rates and haplotype frequencies were provided by the HapMap Project (53-55).

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Figure 1A

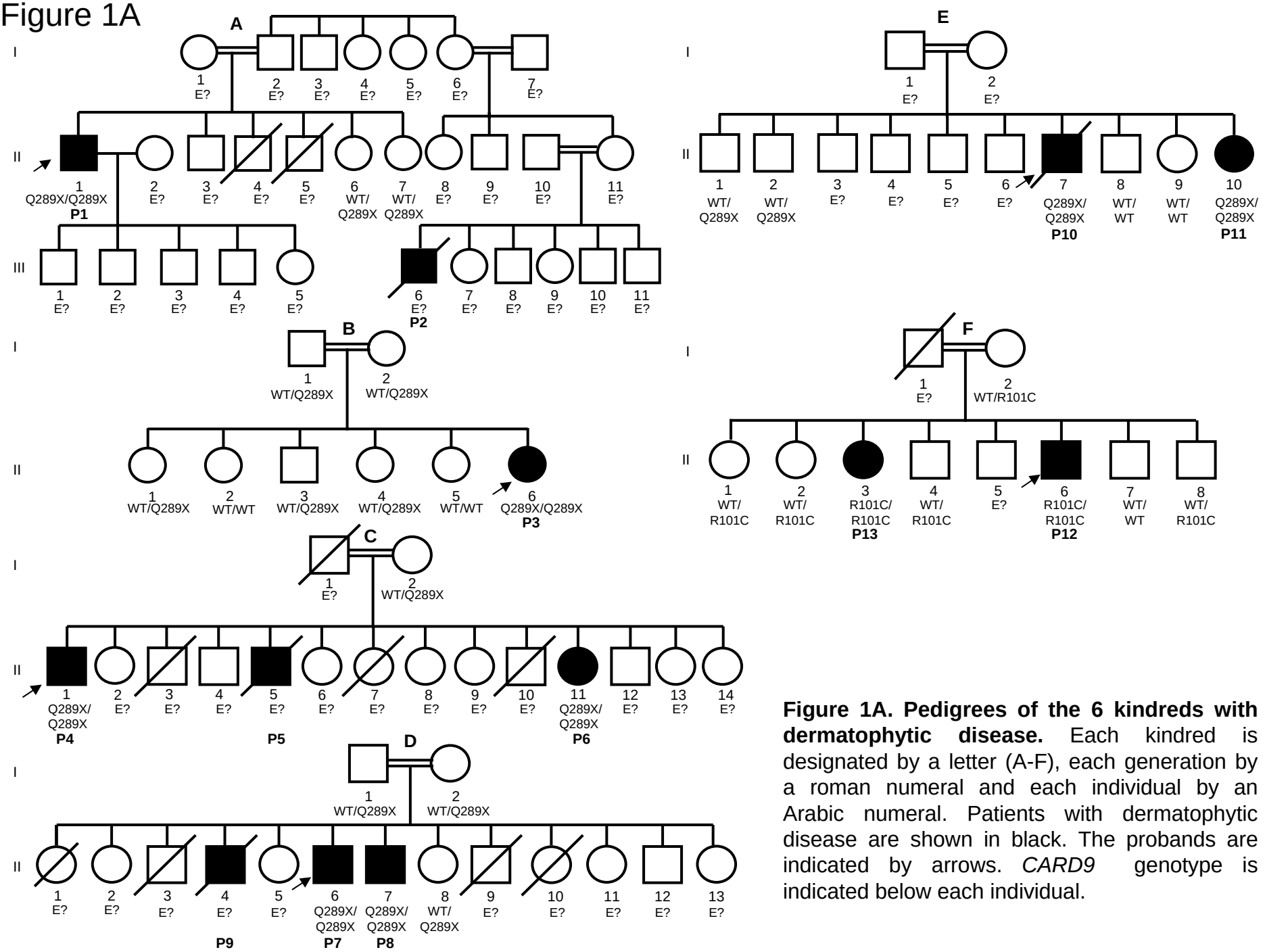

Figure 1A. Pedigrees of the 6 kindreds with dermatophytic disease. Each kindred is designated by a letter (A-F), each generation by a roman numeral and each individual by an Arabic numeral. Patients with dermatophytic disease are shown in black. The probands are indicated by arrows. *CARD9* genotype is indicated below each individual.

Figure 1 B

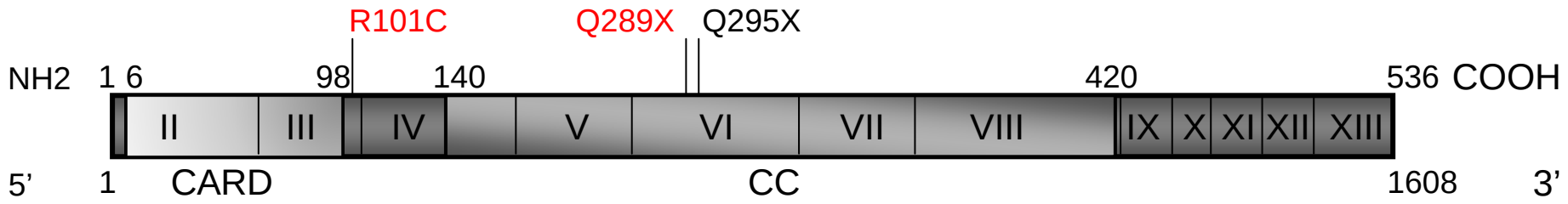

Figure 1B. *CARD9* mutations. The human *CARD9* isoform 1 is shown, with its known pathogenic mutation. Coding exons are numbered with roman numerals. The regions corresponding to the coiled coil domain (CC) and the CARD domain are indicated. Mutations associated with dermatophytic disease are indicated in red.

Figure 2A

Figure 2A. Clinical features of patients. Clinical phenotype of patients: P1 (A-D), P2 (E-F), P3 (G-H), P4 (I-L), P7 (M,N), P8 (O,P), P10 (Q-T), P12 (U,V)

Figure 2B

Figure 2B. Radiological features of patients. Foot MRI fat sat: hypertrophy of toe soft tissues from patient P12 (W, X, Y)

Figure 3

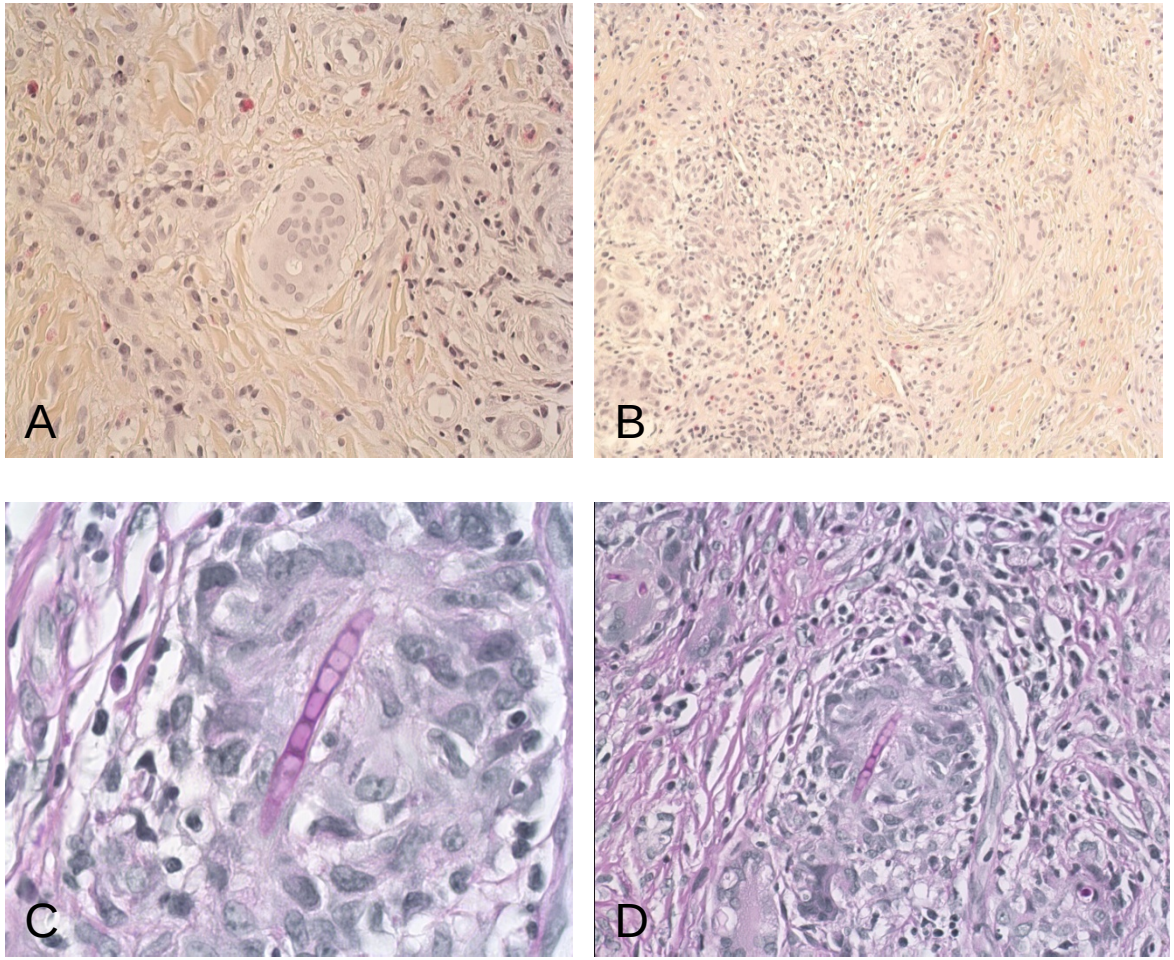

Figure 3. Histology of skin biopsy specimen from patient P13. There is a massive epithelioid granuloma infiltrating the dermis, as shown by hematoxylin-eosin staining (A,B), whereas PAS staining reveals the presence of hyphae consistent with the presence of dermatophytes in its center (C,D)

Figure 4

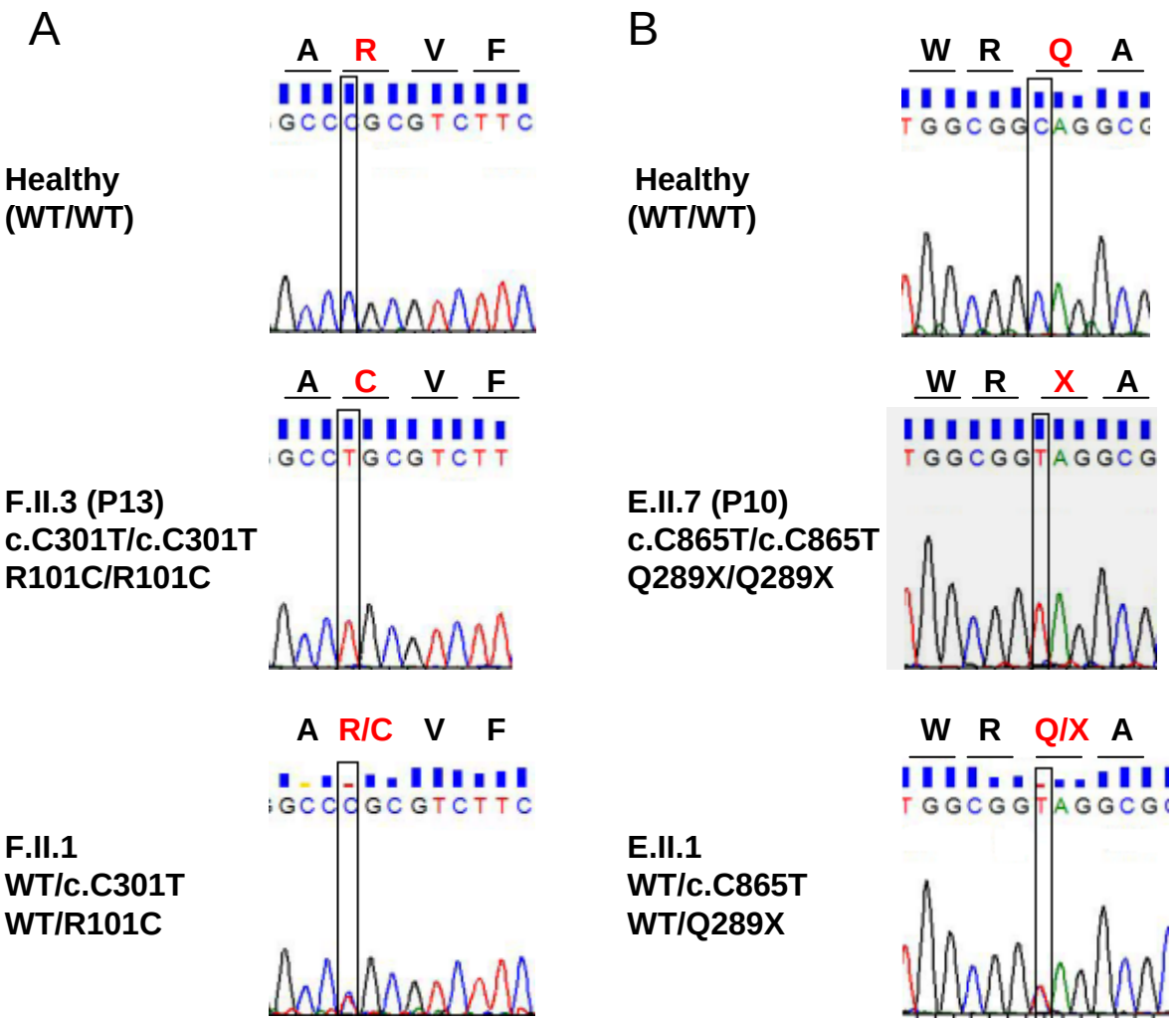

Figure 4: Identification of missense and nonsense mutations in *CARD9*. A. Sanger sequencing reads for a homozygous WT/WT healthy individual (top), for P13, with a homozygous missense mutation in exon 3 of *CARD9* (*c.C301T*) resulting in the amino-acid substitution R101C/R101C (middle) and for the healthy individual F.II.1, with a heterozygous mutation WT/R101C (bottom). **B.** Sanger sequencing reads for a homozygous WT/WT healthy individual (top), for P10, with a homozygous nonsense mutation in exon 6 of *CARD9* (*c.C865T*) leading to a premature stop codon Q289X/Q289X (middle), and for the healthy individual E.II.1, with a heterozygous mutation (WT/Q289X)

Figure 5

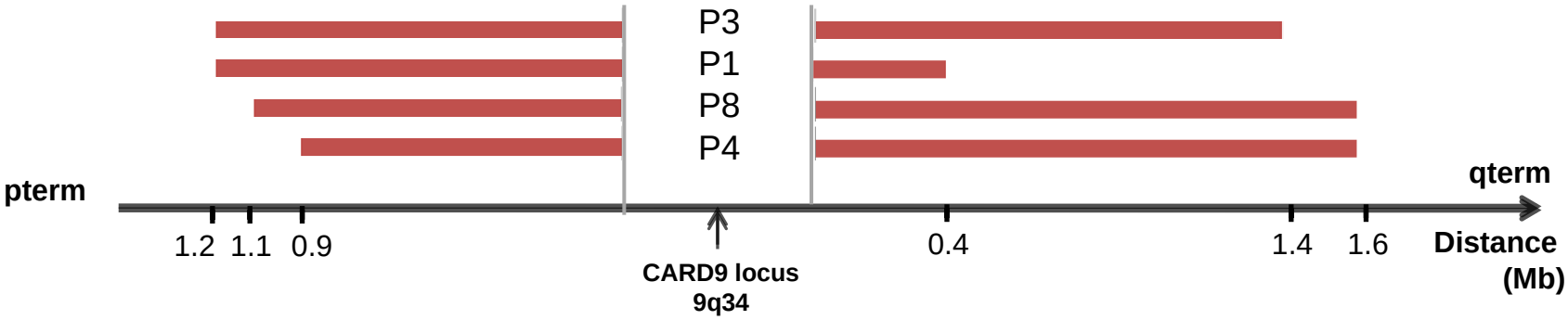

Figure 5. Haplotype common to the 4 unrelated patients carrying the homozygous Q289X mutation of the *CARD9* gene. An analysis of SNP Array 250K data showed that the patients carrying the Q289X mutation had a common haplotype around the CARD9 locus.

Patient	Age at onset, y	Age at last follow-up, y	Sex	Country of origin	Organ involvement	Fungus	Status	CARD9 mutation	Ref
P1, A-II1	6	75	M	Algeria	Skin, scalp, nails, LN	*T. violaceum*	Alive	Q289X/Q289X	(4)
P2, A-III6	2	29	M	Algeria	Skin, scalp, nails, LN, brain	*T. violaceum*	Dead	E?	(9, 57)
P3, B-II6	9	40	F	Algeria	Skin, scalp, nails, LN	*T. violaceum*	Alive	Q289X/Q289X	(58)
P4, C-II1	8	56	M	Algeria	Skin, scalp, nails	*T. violaceum*	Alive	Q289X/Q289X	(8)
P5, C-II5	8	37	M	Algeria	Skin, scalp, nails, LN	*T. violaceum*	Dead	E?	(8, 59)
P6, C-II11	8	ND	F	Algeria	Nails	*T. violaceum*	Alive	Q289X/Q289X	
P7, D-II6	19	43	M	Algeria	Skin, scalp, nails, LN	Dermatophyte *	Alive	Q289X/Q289X	
P8, D-II7	21	40	M	Algeria	Skin, perineum, scalp	Dermatophyte *	Alive	Q289X/Q289X	
P9, D-II4	NA	28	M	Algeria	Skin, scalp	Dermatophyte *	Dead	E?	
P10, E-II7	NA	39	M	Algeria	Skin, scalp, LN	*T. violaceum*	Dead	Q289X/Q289X	(60)
P11, E-II10	NA	NA	F	Algeria	Nails	Dermatophyte *	Alive	Q289X/Q289X	
P12, F-II6	16	40	M	Morocco	Skin, bone, LN, nails,	*T. rubrum*	Alive	R101C/R101C	
P13, F-II3	NA	49	F	Morocco	Scalp, nails	Dermatophyte *	Alive	R101C/R101C	

Table 1. Description of the 13 patients with dermatophytic disease ; y: year, NA: not available, E?: no DNA available, LN: lymph nodes, *: hyphae on biopsies or scrapping

	N (%)
Median age [years]	40 (range: 28-75)
Male	9 (64)
Country of origin	
Morocco	2
Algeria	11
Median age at first symptoms [years]	8 (range: 2-21)
First symptoms	
Severe or recurrent tinea capitis	10
Severe or recurrent tinea corporis	7
Onychomycosis	6
Presentations in adulthood	
Lymph node involvement	7
Central nervous system invasion	1
Local organ invasion (bone, digestive tract)	2
Associated infection: thrush	4
Death	4
Median age at death [years]	31.5 (range: 28-39)
Dermatophyte identified	
T. rubrum	2
T. violaceum	6
Histology (n=9)	
Granuloma	7
Necrosis	5
Hyphae on biopsy	9
High IgE levels	3/3
Hypereosinophilia	6/6
Lymphocyte subset	
Normal $CD4^+$ T lymphocyte susbset	3/3
Normal $CD8^+$ T lymphocyte subset	3/3
Normal B lymphocyte subset	3/3
Normal NK lymphocyte subset	3/3

Table 2. Characteristics of the 13 patients with dermatophytic disease

Résumé

Mon projet de thèse a consisté en l'identification et la caractérisation moléculaire et immunologique de patients présentant une susceptibilité accrue aux infections fongiques par *Candida* sp. dans le syndrome Mendélien de candidose cutanéo-muqueuse chronique (CCMC).

La CCMC est caractérisée par des infections persistantes ou récurrentes de la peau, des ongles et des muqueuses par les champignons *Candida*, principalement *C. albicans.* Elle est fréquemment associée à d'autres infections opportunistes dans certaines immunodéficiences primaires ou acquises, ou bien elle peut être associée à un tableau auto-immun. La CCMC peut finalement être isolée (CCMCi) sans autre tableau clinique sévère: la plupart des cas rapportés sont sporadiques, mais il existe également des cas familiaux avec une hérédité mendélienne autosomique principalement dominante (AD) ou plus rarement récessive (AR).

Basés sur les données de la littérature, qui démontrent un rôle majeur de l'immunité dépendante des IL-17s dans la résistance aux infections mucocutanées vis-à-vis de *C. albicans* et nos résultats récents, qui démontrent un défaut de cette immunité dans certaines immunodéficiences primaires associées à une CCMC [les syndromes AD-HIES et AR APS-1, ainsi que chez les patients déficients en CARD9, nous avons émis l'hypothèse que parmi les patients atteints de CCMCi, certains pourraient présenter un défaut génétique affectant spécifiquement l'immunité IL-17-dépendante.

Au début de ma thèse, j'ai participé à l'identification des deux premières étiologies génétiques de la CCMCi : le défaut autosomique récessif (AR) complet en IL-17RA et autosomique dominant (AD) en IL-17F. Plus récemment, j'ai identifié la troisième et la plus fréquente étiologie génétique de la CCMC par l'identification de mutations gain de fonction dans le gène STAT1 suite à une approche explorant l'ensemble du génome (séquençage de l'ensemble des exons). Ces mutations engendrent une « hyper-réponse » aux interférons de type I et II et à l'IL-27 qui inhibent la différentiation des lymphocytes T sécréteurs d'IL-17, impliqués dans l'immunité mucocutanée vis-à-vis de *C. albicans* chez l'homme.

En conclusion, nous avons identifié, en 2011, des trois premières étiologies génétiques de la CCMCi, avec les défauts AR en IL-17RA, AD en IL-17F et des mutations gain-de-fonction de *STAT1,* toutes associées à un défaut de l'immunité dépendante de l'IL-17. Des mutations gain-de-fonction de *STAT1* représentent à ce jour la cause génétique la plus fréquente de la CCMCi avec au total 94 patients rapportés dans la littérature depuis 2011. Nous avons ainsi démontré que la CCMCi est une immunodéficience primaire, associée à un défaut de l'immunité réalisée par les IL-17s. Ces travaux ont des implications majeures dans le domaine immunologique avec la description et la caractérisation des mécanismes biologiques impliqués dans l'immunité protectrice spécifique de *C. albicans* et une meilleure compréhension des mécanismes physiopathologiques associés à une susceptibilité accrue aux infections fongiques, dans des conditions naturelles d'infection ; et dans le domaine médical, avec la possibilité de diagnostics moléculaires, un conseil génétique en cas de diagnostic positif, une meilleure prise en charge des patients.

Summary

My project consists in the molecular and immunological identification and characterization of patients with increased susceptibility to fungal infections with *Candida* sp. suffering from the Mendelian syndrome of chronic mucocutaneous candidiasis (CMC).

CMC is characterized by persistent or recurrent infections of the skin, nails and mucosae by *Candida* fungi, especially *C. albicans*. CMC is frequently associated with other opportunistic infections in some acquired or primary immunodeficiencies, or can be associated with autoimmune disorders. Finally, CMC may be present as an isolated form (chronic mucocutaneous candidiasis disease or CMCD) without any other severe infectious or autoimmune clinical manifestation: most reported cases are sporadic, but there are also familial cases with autosomal dominant (AD) or recessive (AR) Mendelian inheritance. Based the literature, which demonstrated a major role of IL-17 cytokines in mucocutaneous immunity with *C. albicans*, and our recent results, which show an impairment of IL-17 immunity in some primary immunodeficiencies associated with CMC (AD-HIES syndrome, AR APS-1, and CARD9-deficient patients), we hypothesized that among CMCD patients, some might have a genetic defect affecting specifically the IL-17-dependent immunity.

At the beginning of my PhD, I participated in the identification of the first two genetic etiologies of CMCD: complete AR IL-17RA and partial AD IL-17F deficiencies. More recently, I identified the third and most common genetic etiology of CMCD by identifying gain of function mutations in the *STAT1* gene following an approach exploring the whole genome (sequencing of all exons). These mutations are responsible for a "hyper-response" to type I and II interferons and IL-27, which inhibit the differentiation of IL-17-producing T cells. Impaired IL-17 immunity results in reduced mucocutaneous defenses against *C. albicans* in humans.

In conclusion, we have identified in 2011, the first three genetic etiologies of CMCD with AR IL-17RA and AD IL-17F deficiencies and gain-of-function *STAT1* mutations, all associated with an impaired IL-17-dependent immunity. Gain-of-function *STAT1* mutations represent the most frequent genetic cause of CMCD with a total of 94 patients reported in the literature since 2011. We have shown that CMCD is a primary immunodeficiency associated with inborn errors of IL-17 immunity. This work has important implications in the field of immunology with the description and characterization of the biological mechanisms involved in protective immunity specific to *C. albicans* and a better understanding of the pathophysiological mechanisms associated with increased susceptibility to fungal infections in natural conditions of infection, and in the medical field, with the possibility of molecular diagnostics, genetic counseling for a positive diagnosis, and a better follow-up of the patients.